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**EXPRESSION AND FUNCTION OF NG2/CSPG4
IN HUMAN CHONDROCYTES**

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DOCTOR OF PHILOSOPHY
PATHOLOGY
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Declaration

I hereby declare that this thesis has been composed by me and has neither been presented nor accepted in any previous application for a degree. All work presented in the thesis was carried out by myself. All sources of information have been acknowledged by reference.

Nuor Jamil

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Abstract

Introduction: NG2/CSPG4 is a unique transmembrane chondroitin sulphate proteoglycan molecule expressed as a core protein and a chondroitin sulphate proteoglycan (CSPG) up to 400kD. NG2/CSPG4 mediates the communication between the extracellular and intracellular compartments through interactions with collagen VI, growth factors and the actin cytoskeleton. NG2/CSPG4 affects cell migration, spreading, apoptosis and proliferation processes. NG2/CSPG4 has been shown to be expressed in developing and adult cartilage where less is known of its function. I tested the hypothesis: NG2/CSPG4 is an important regulator of chondrocytes function and has the potential to be a therapeutic target for treatment of diseases of cartilage such as osteoarthritis and chondrosarcoma. To do this, I had the following aims: 1) investigate whether different types of chondrocytes show variation in the form or distribution of NG2/CSPG4 expression and 2) through a knockdown approach develop a model to study the functional roles of NG2/CSPG4 in human chondrocytes.

Materials and Methods: JJ012, a chondrosarcoma cell line, chondrocytes derived from human articular cartilage and C20/A4 an immortalised chondrocyte cell line were used. NG2/CSPG4 expression was investigated by RT-PCR western blotting, flow-cytometry and immunocytochemistry. NG2/CSPG4 interaction with Golgi complex and endoplasmic reticulum (ER) was assessed by double immunofluorescence. Biochemical interactions were assessed by immunoprecipitation and mass spectroscopy. For NG2/CSPG knockdown, a viral transduction method was carried out using 5 different constructs. Different functional roles of NG2/CSPG4 were investigated. The role of NG2/CSPG4 in gene regulation was studied by shRNA knockdown of NG2/CSPG4 in JJ012 cells and RTPCR.

Results: NG2/CSPG4 mRNA was detectable in all cells tested. Western blotting showed expression of only a 270kD core protein in JJ012 and C₂₀A₄ cells. Using two different anti NG2/CSPG4 antibodies human OA chondrocytes were seen to express multiple molecular weight forms differentially recognised with and without chondroitinase ABC pre-treatment. Expression of NG2/CSPG4 in JJ012 cells was predominantly membrane associated whilst in OA chondrocytes and C₂₀A₄ cells, additional, predominant punctuate cytoplasmic distribution was evident. In OA chondrocytes NG2/CSPG4 co-localised with the Golgi complex and ER. Immunoprecipitation and mass spectrometry data demonstrated associations between NG2/CSPG4 and both collagen VI and thrombopoietin in OA chondrocytes. A model of NG2/CSPG4 gene knockdown was achieved in JJ012 chondrosarcoma cell line, known as B3. B3 cells spread more and migrate less than JJ012 cells; with a significant difference observed in migration (after 10hours: the closed area was 81.4% for JJ012 and 54.6% for B3). There was no difference in cell adhesion to collagen I, II, VI and fibronectin. EGTA inhibited cell adhesion to fibronectin in dose dependent manner with no significant difference

observed between both JJ012 and B3 cells. EDTA reduced adhesion of B3 cells but not JJ012 to fibronectin. A significant difference in cell proliferation was detected with no change in apoptosis. Following NG2/CSPG4 knockdown in JJ012 cells there was no difference in expression of aggrecan, collagen II and SOX-9. In contrast, B3 cells showed a decreased expression of MPP3 and ADAMTS-4, a complete loss of ADAMTS-5 and increased expression of MMP13.

Conclusions: I have identified altered expression and multiple forms of NG2/CSPG4 in different types of chondrocytes and shown association of this molecule with type VI collagen and thrombopoietin. Creation of a chondrocyte cell line that has stable knockdown of NG2/CSPG4 allowed further investigation of NG2/CSPG function in chondrocytes. NG2/CSPG4 knockdown reduced the cellular migration and proliferation and increased the chondrocyte spreading. The adhesion mechanism in JJ012 appears to be calcium dependent. Loss of NG2/CSPG4 induced changes in expression of aggrecanases and MMPs. Altered expression or associations of NG2/CSPG4 with extracellular ligands or intracellular signalling cascades may be important in the pathogenesis of OA by regulating proteolytic activity or apoptosis related pathways. NG2/CSPG4 is a potential therapeutic target in degenerative and neoplastic diseases of cartilage.

CHAPTER ONE

Introduction

1.1 Cartilage

Cartilage is a specialised but ubiquitous connective tissue. It is an avascular tissue that gets its oxygen supply and nutrition by the dynamic flow from the synovial fluids during joint movement. Cartilage is composed predominantly of extracellular matrix, within which chondrocytes are embedded, cells which maintain cartilage matrix structure by continued remodelling activity throughout life. Cartilage is covered by a fibrous perichondrium except at osseous junctions and at synovial surfaces. The main roles of articular cartilage in the joint are to reduce friction at the joint during motion and to distribute the load evenly, which in turn can reduce the stress on the subchondral bone. There are three main types of cartilage which differ in the number and morphology of the constituent chondrocytes and the biochemical composition of the matrix (Poole AR et al 1997; Gardner et al. 1997):

- Hyaline cartilage
- Fibro cartilage
- Elastic cartilage

1.2 Composition of human articular cartilage

Articular cartilage is composed of the extracellular matrix, within which chondrocytes are embedded. Unlike other tissues, such as bone and muscle, articular cartilage has low metabolic activity and its responsiveness to injuries can be detected either microscopically or by metabolic studies. Despite that, articular cartilage has its elaborate and complex structure, where ECM molecules interact with the embedded chondrocytes (Buckwalter et al. 2005).

Articular cartilage can be divided into four layers: superficial, transitional, radial and the calcified cartilage layer (Wong and Carter 2003). There are different distributions of collagen network, types and the amounts of proteoglycans in these cartilage layers (Burgeson et al. 1982; Poole et al. 2001; Eyre 2002). The organisation of the collagen and the chondrocytes within cartilage layers are different. While in the superficial layer, collagen fibres and chondrocytes are arranged in a parallel line to the cartilage surface, collagen fibres are organised randomly within the deeper layers and provide a support to the chondrocytes. In the deepest layers, collagen fibril is distributed vertically and inserted into the underlying subchondral bone (Silver et al. 2001). The structural difference in the cartilage layers is well correlated to the distribution of pressure, deformities and the pressure induced fluid flow across the cartilage as shown *in vivo* and *ex vivo* experiments (Burgeson et al. 1982).

1.2.1 The extracellular matrix

The main constituent of the extracellular matrix of the cartilage is water (65 to 80%). The rest of the ECM is composed of collagens (15-25%) and proteoglycans and non collagenous proteins (10%) The ECM molecules of the human articular cartilage are secreted by chondrocytes. The chondrocytes are embedded in an extensive ECM composed from macro fibrillar collagen network, mixed with micro filamentous network of non collagenous materials (proteoglycans). Most of proteoglycan molecules exist as aggregating molecules composed from GAGs attached non- covalently to core protein. The structure and composition of the articular cartilage proves that it has very special mechanical properties that reflects its tensile strength, provided by collagens and the compressive stiffness by proteoglycan aggrecan, together with cell-matrix interactions by non collagenous proteins (Poole et al. 2001).

1.2.1.1 Cartilage collagens

Articular cartilage has its distinct collagen fibrillar network. Collagens fibres are extensively cross-linked within the cartilage and have a characteristic distribution within different zones of the articular cartilage. Each type of collagen has its characteristic phenotype and distribution within the cartilage. The main type of collagen in articular cartilage is type II collagen. Minor collagens present in the articular cartilage are: types VI, IX, X, XI (Eyre 2002; Eyre 1991)

Type II collagen

Type II collagen is the single most abundant protein in normal articular cartilage comprising 85 -95 % of the collagen present. Type II collagen is a fibrillar collagen and is different from that found in other connective tissues such as in skin or bone where type I collagen predominates. It consists of three identical $\alpha 1$ chains in a helical form. Type II collagen is synthesised in a precursor form, procollagen, which contains propeptides needed for proper fibril assembly. Following secretion these propeptides are removed and fibril formation occurs. Type II collagen fibrils are composed of 1.5 nm, 300 nm long tropocollagen molecules arranged in a quarter stagger. These collagen molecules are stabilised by hydroxypyridinium cross links which result after secretion. Mutation in type II collagen has been identified as the cause of a number of rare chondrodysplasias and is implicated in some familial forms of osteoarthritis.

Type VI collagen

Type VI collagen is a short chain collagen which shows preferential expression in the pericellular matrix of cartilage where it forms a filamentous network. It is a major component

of intervertebral disc cartilage where it accounts for about 20% of total collagen. It is known to bind to hyaluronic acid but in general its function in cartilage is unknown. Interactions with specific receptors, including integrins, expressed by chondrocytes may allow regulation of synthetic or anabolic activity. Alternatively it may function as a bridging molecule between chondrocytes and collagen fibrils in cartilage matrix.

Type IX collagen

Type IX collagen is a non-fibrillar collagen which is covalently bound to fibrils of type II collagen in an antiparallel fashion. It accounts for about 1% of collagenous protein in adult cartilage and up to 10% in foetal cartilage. Type IX collagen supports the tensile properties of type II collagen and may also function to limit fibril diameter.

Type XI collagen

Type XI collagen is also closely associated with type II collagen and makes up about 3% of mature cartilage collagen. It is found within type II collagen fibrils and may play a role in fibril formation.

Type X collagen

Type X collagen is a short chain collagen which has limited expression in cartilage. Type X collagen is normally expressed in the hypertrophic zone of growth plate cartilage. It is however absent from normal articular and intervertebral disc hyaline cartilage but is expressed in these tissues in disease. Type X collagen interacts with type II collagen and

appears to be important in endochondral ossification potentially with roles in matrix calcification.

1.2.1.2 Cartilage proteoglycans and other macromolecules

Proteoglycans are composed of core protein, with one or more glycoaminoglycan chains attached to it (Roughley and Lee 1994). Glycoaminoglycans within the cartilage include: hyaluronic acid, chondroitin sulphate, keratan sulphate and dermatan sulphate. The concentration of these molecules is affected by patient age, diseased condition, and exposure to injuries and is also different within the cartilage areas of the same individual (Buckwalter et al. 2005).

Aggrecan is the most predominant cartilage aggregating proteoglycans and it is crucial for the proper function of the articular cartilage to resist compressive loads. About 90% of the proteoglycan components of the articular cartilage are aggrecan, while large non aggregating proteoglycans constitute 10% and only 3% for small non aggregating proteoglycans. Aggrecan is composed of protein core with three functional globular domains (G1-3), which are heavily keratan and chondroitin sulphated. The major extended part of the protein core, containing more than half of the amino acids, is situated between the G2 and G3 domains and is substituted with keratan sulphate and chondroitin-4 and chondroitin-6 sulphate glycosaminoglycan chains. Keratan sulphate is a repeating disaccharide of D-galactose and N-acetylglucosamine. Chondroitin sulphate consists of disaccharide repeats of glucuronic acid and N-acetylgalactosamine. Some 30 keratan sulphate side chains are attached in a region immediately adjacent to G2. Keratan sulphate content changes with age. About 100 chondroitin sulphate side chains are attached to the protein core in 2 sub regions CS-1 and CS-2 each containing amino acid repeats of different nature. Aggrecan also contains smaller

amounts of O-glycosidically and N-glycosidically linked oligosaccharides (Hardingham 1992). The aggregate formation is very important to keep the proteoglycans in their location within the cartilage, prevents their displacement during cartilage deformation as well as stabilises the relationship between the proteoglycans and the collagen network. Examples of non aggregating proteoglycans expressed in the cartilage include: the cell surface syndecans and glypican, the small leucine-rich proteoglycans decorin, biglycan, fibromodulin, lumican and epiphykan and the basement membrane proteoglycan, perlecan (Buckwalter et al. 2005). The proteoglycans are very important in cartilage degeneration and chondrogenesis (Knudson and Knudson 2001).

1.1.2 Chondrocytes

Chondrocytes are the specialized mesenchymal cells of the articular cartilage and constitute 10% of articular cartilage components. They secrete the extracellular matrix (ECM) that forms cartilage, which separate the chondrocytes from each other. Chondrocytes secrete large quantities of type II collagen and proteoglycan and different proportions of type VI, IX, X and XI collagens. Chondrocytes are different in their phenotypic characters in different cartilages and also between chondrocyte subpopulations in different parts of the same type of cartilage. A variety of local environmental and humoral factors including exposure to mechanical forces influences the regulation of the chondrocyte phenotype, which ultimately results in the differential expression of a variety of cell surface receptors for cytokines and extracellular matrix molecules which regulate chondrocyte function. Chondrocytes possess cell surface receptors for extracellular matrix molecules including members of the integrin super family, which include receptors for collagen, and CD44 a receptor for hyaluronic acid. Interactions between chondrocytes and the extracellular matrix in cartilage are essential to maintain the cartilage structure in both normal and diseased conditions. Integrins and other

cell adhesion molecules are essential mediators of cell-matrix interactions through their signal transduction pathways from the exterior of the chondrocyte genome, to the inside of the cells, where they regulate the cartilage matrix turnover (Gardner et al. 1997).

The adult articular chondrocytes are organised in different patterns in different cartilage layers, embedded in an extensive network of extra cellular matrix (composed of collagenous and non collagenous constituents) (Poole et al. 2001). Chondrocytes are inactive cells with a very low synthetic activity and no observed mitotic divisions in the resting phase. Upon cell activation, they begin to secrete the extra cellular matrix around themselves and are then being referred as chondrocytes. Spaces contain these chondrocytes are called lacuna (Muir 1995).

The primary articular chondrocytes in monolayer can preserve the phenotype until they are passaged. Immortalization of the primary human articular chondrocytes with viral oncogenes produces a cell line which has a high proliferative capacity and property of differentiated chondrocytes (Alema et al. 1985; Thenet et al. 1992; Mallein-Gerin and Olsen 1993). Like all other cell types, chondrocytes can undergo malignant transformation but their unique environment means that chondrosarcoma pose particular problems for treatment and clinical management.

1.2 Osteoarthritis (OA)

Osteoarthritis (OA) is a degenerative disease of the joint. It occurs as a result of imbalance between the degenerative and synthetic processes (Mollenhauer and Erdmann 2002). OA is a multifactorial disease, with various risk factors involved in its pathogenesis: age, obesity, trauma and overuse (Zhang and Jordan 2010). It is more useful to classify these factors into: mechanical (such as: overuse, trauma, and joint surgery) or biological (such as: genetic risk and metabolic disorders)(Muthuri et al 2011; Loughlin 2010; Toivanen et al 2010 and Zhang and Jordan 2010). OA process can occur after mechanical or biological factors applied to the joint and it usually starts in one component of the cartilage (Kamekura et al. 2005; Blair-Levy et al. 2008). However, the end results would be the involvement of both articular cartilage and the subchondral bone (Madry et al. 2010). This raises the possible interaction between the articular cartilage and the subchondral bone in the development of the OA process.

1.2.1 Pathogenesis of osteoarthritis

Although OA is regarded as a mechanically induced disease, however, both mechanical and biological factors are interlinked and participate in OA pathophysiology. Direct mechanical injury to the articular cartilage or misalignment of the peri articular structures such as nerve, muscle and ligament usually cause stress overload on the cartilage -bone unit (Brandt 2004; Kamekura et al. 2005). Through mechanotransduction process, a more extensive damage to the articular cartilage and subchondral bone can occur, whereby chondrocytes convert these mechanical signals into a biological responses and changes in the genetic profiles of the articular chondrocytes are induced (Zhu et al. 2011; Brandt 2004).

Although biological factors such as genetic predisposition and biochemical changes in the articular cartilage can aggravate the articular cartilage response to mechanical overload, the roles of these factors has not been clearly defined (Schroeppel et al. 2011).

Anabolic and catabolic changes in the articular cartilage have been reported following mechanotransduction. Chondrocytes have receptors to respond to mechanical stimulation. In the early stages of OA, chondrocytes respond to these changes and start to increase the quantities of proteoglycans and collagen produced, as an attempt to regenerate the cartilage matrix (Poole et al. 2001). As the OA process progresses, gross damage to articular cartilage becomes evident and the cartilage starts to lose its normally smooth surface, where it becomes rough or eroded. In addition to the anabolic response to the mechanical stimulation by the chondrocytes, chondrocytes participate in the catabolic process in OA by increasing the production of the inflammatory cytokines, which are also produced by other joint tissues (Goldring and Goldring 2007). Chondrocytes express chemokines and chemokine receptors which are important in cartilage metabolism and the OA process (Borzi et al. 2004). In the OA process, although there is a production of different cytokines and chemokines by chondrocytes, they can also respond to the chemokines and cytokines produced by other cartilage structure, such as the synovial fluid and subchondral bone (Goldring and Goldring 2007).

1.2.2 Signalling pathways involved in osteoarthritis

Signalling pathways, such as Wnt/beta-catenin, nuclear factor-KB, Cyclooxygenase (COX), ERK1/2, p38 MAPK, and SAPK/ERK kinase-1 (SEK1) of the JNK pathway, have been involved in the mechanical stimulation process in articular cartilage (Kreke et al. 2008; MacLean et al. 2008). Furthermore, many mediators are involved in this process, which

either promotes catabolic and/or remodelling processes in cartilage. Examples of these mediators are: a reduction in the matrix proteins production (aggrecan and collagen II), up regulation of the pro-inflammatory cytokines, such as [interleukin-1 (IL)-1, IL-6, IL-8, IL-17 and IL-18], increase in the production of proteases such as Matrix Metalloproteinase (MMP)-2, MMP-3, MMP-13 and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4 and (ADAMTS)-5 (MacLean et al. 2008), as well as reactive oxygen species (ROS; such as NO, superoxide, hydrogen peroxide, and peroxynitrite) (Howard et al. 2008). Moreover, it has been shown that there is a change in the methylation in the promoter regions of SOX-5, 9, under mechanical stress in a surgically induced OA model (Kim and Im 2011).

1.3 Chondrosarcoma

Chondrosarcomas are a heterogeneous group of neoplasms characterised by the production of cartilage matrix by the tumour cells (Gelderblom et al. 2008). These tumours possess a low percentage of dividing cells and increased amount of extra cellular matrix, rendering them relatively radio- and chemo resistant (Bjornsson et al. 1998). The majority of these tumours are slowly growing and rarely metastasise.

Conventional chondrosarcoma accounts for approximately 85% of human chondrosarcoma. The majority of conventional chondrosarcoma are primary central as they originate in the medullary cavity of the bone. Fifteen per cent of them are regarded as secondary peripheral, where they are located peripherally in the bone and they usually develop from the malignant transformation of the cartilage cap of the pre-existing osteochondroma, one of the benign cartilage tumours. Less than 1% of chondrosarcoma originate from the peripheral surface of the bone, usually the periosteum, and therefore, they are called periosteal chondrosarcomas (Bertoni et al. 2002, World Health Organization Classification of the Tumours). There are

other types of chondrosarcomas which are rare: Dedifferentiated, clear cell and mesenchymal chondrosarcomas. Dedifferentiated chondrosarcoma is a distinct variety of chondrosarcoma in which a well-differentiated low-grade cartilage tumour is juxtaposed to a high-grade non-cartilaginous sarcoma. These are aggressive lesions with poor prognosis (Grimer et al. 2007; Milchgrub and Hogendoorn et al 2002, World Health Organization Classification of Tumours).

Central and peripheral chondrosarcomas are histologically more or less the same. Each one is classified into three different grades. Grade I chondrosarcoma is characterised by a low percentage of cells, high percentage of hyaline cartilage production and very rarely metastasises (Bjornsoon et al 1998). However, high grade chondrosarcomas have a high percentage of cell number, mucomyxoid matrix, high mitosis and 70% of the cases have already metastasised by the time of diagnosis (Evans et al. 1977). Recurrent chondrosarcoma have a higher grade of malignancy, risk of tumour progression from low to high grade and even differentiation, with severe adverse prognosis than the original neoplasm (Evans et al. 1977; Bjornsson et al. 1998) .

Over the last decade, more understanding of the molecular pathways involved in the pathogenesis of chondrosarcoma have been identified, such as the role of tumour suppressor and oncogenic pathways as well as signal transduction mechanisms. All of these can serve as therapeutic targets for treating patients with chondrosarcoma.

One important tumour suppressor pathway is p53. It has been found that p53 mutation is associated with high grade chondrosarcomas and it is also involved in tumour progression (Terek et al. 1998; Papachristou et al. 2006). Rb1 (retinoblastoma) is localised to 13q14, and

is a nuclear phosphoprotein with DNA binding activity, is also reported to be involved in chondrosarcoma. It has been found that the loss of 13q is associated with local recurrence (Yamaguchi et al. 1996) and loss of its heterozygosity has been reported in 67% of cases (Papachristou et al. 2006). The INK4 family of proteins (p15, p16, p18, p19) are cyclin dependent kinase inhibitors that bind and reduce the activity of CDK4/CDK6 kinases and the CIP/KIP proteins (p21, p27, p57). The INK4A TSG (DDKN2) maps to 9p and encodes p16 and p14, which inhibit formation of cyclin D1-3/CDK4-6 and cyclin E/CDK2 complexes and maintain the under-phosphorylated state of Rb1 (Swanton 2004). Sandberg and his colleagues in 2004 showed that there are p16 mutations and CDKN2A methylations in chondrosarcoma (Sandberg 2004).

For the oncogenic pathways, the myc proto-oncogenes (type C) are amplified in high but not low grade chondrosarcoma and they are associated with tumour progression (Morrison et al. 2005). AP-1 is a homo-(Jun–Jun)/ heterodimeric (Jun–Fos) transcription factor involved in the expression of genes which are associated with the osteoblasts/chondroblasts growth and differentiation. It has been reported that c-Jun and c-Fos are highly expressed in chondrosarcoma but not in benign cartilage tumours like enchondroma, raising the importance of these genes in the development of the chondrosarcoma (Papachristou et al. 2005).

1.4 NG2/CSPG4 structure

NG2 / CSPG4 is a transmembrane chondroitin sulphate (CS) proteoglycans, expressed by cells as a core protein of 250-300kD and a chondroitin sulphate proteoglycan (CSPG) of molecular weight greater than 400kD. NG2/CSPG4 is also known as NG2; MCSP; MCSPG;

MSK16; HMW-MAA; MEL-CSPG (Pub med, gene search). NG2/CSPG4 has few similarities to other glycoproteins, indicating that NG2 is a novel species of integral membrane protein. Comparison of the amino acid sequences of NG2/CSPG4 to other proteoglycan proteins reveals that NG2 is the rat homologue of human melanoma proteoglycan and the mouse AN2 protein (Pluschke et al. 1996; Schneider et al. 2001). NG2/CSPG4 contains a very large 2225 amino acid extracellular domain, a 25 amino acid residues transmembrane domain, and a relatively short 76 amino acid cytoplasmic domain (Nishiyama et al. 1991). It is an atypical membrane intercalated molecule which is impossible to isolate in the absence of detergent (Nishiyama et al. 1991). Sequence analysis of the NG2/CSPG4 core protein showed that it is composed of 8,071 nucleotides with an open reading frame coding of 2,325 amino acids. The 3' untranslated regions consist of 1,027 nucleotides and contain two polyadenylation signals (AATAAA) 24 and 18bp upstream of the poly (A) tail. In the coding region, sequences of two of the CNBr cleavage products have been identified. The amino-terminal amino acid sequence determined from the purified NG2 is also found, beginning at residue 30, which is identical to the amino-terminal sequence determined for the core protein of human melanoma proteoglycans. The nucleotide sequence around the ATG at position 70 represents the consensus sequence for the translation initiation site. The 5'69 by of the cDNA contribute to the 5' untranslated region (Nishiyama et al. 1991).

1.4.1 The extracellular domain of NG2/CSPG4

The extracellular domain of NG2/CSPG4 contains three domains: domain1 (D1), domain 2(D2) and domain 3(D3). D1 and D3 are globular in shape (8 cysteine containing domains) and connected by a more central extended domain 2 (nine serine-glycine-rich domain) (Burg et al 1997). Domain 1 is stabilised by the disulfide bonds (Nishiyama et al. 1991) and has two

laminin G-type motifs, which may be important for ligands binding (Stegmuller et al. 2002). Domain 2 has 950 amino acids (residues 641-1590), which is devoid of cysteine residues. This domain has a long chondroitin sulphate chain site at ser-999 position. It was found that the amino acid sequences at the serine residues at position 998 and 1342 is a consensus sequence, identified as chondroitin sulphate attachment sites by Zimmermann and Rouslahti in 1989 and Bourdin and his colleagues in 1997 (Bourdon et al. 1987; Zimmermann and Ruoslahti 1989). So, this domain is considered as a putative glycosaminoglycan attachment domain. The attachment of glycosaminoglycan chains is developmental and cell-type specific (Schneider et al. 2001). Given that NG2/CSPG4 is present on multiple cell types and at different stages of development, it is likely that NG2/CSPG4 possesses more than one cellular role and has more than one interacting partner. In addition, the helical amino terminal portion of domain 2 possesses a site for collagen V and VI attachments (Nishiyama et al. 1991; Burg et al. 1997; Stallcup and Dahlin-Huppe 2001).

There are surface ligands for NG2. Matrix metalloproteinase (MMPs) appear to represent a type of cell surface receptor for NG2. NG2 interacts with membrane type- 3 membrane metalloproteinase (MT3-MMP) and forms a complex which is critical for the ability of melanoma cells to invade type I collagen containing matrix (Iida et al. 2001). Furthermore, NG2 ectodomain interacts with the kringle domain of some proteins, such as plasminogen. The plasminogen is converted into active plasmin, where the latter forms a complex with NG2. This complex interaction between plasmin and NG2 enhances the motility process of both normal and neoplastic cells (Goretzki et al. 2000). The anti-angiogenic protein, Angiostatin, is the proteolytic fragment of plasminogen, retains four of the kringle domains and therefore, exhibits strong binding to NG2. Angiostatin interaction with NG2 inhibits the

proliferation of endothelial cells, highlighting another mechanism by which NG2 may regulate angiogenesis (Goretzki et al. 2000).

In all above examples, NG2 did not serve as a primary signalling molecule. However, NG2 is involved in the regulation of function of other cellular factors or receptors in different cells. The most characterised interaction for NG2 is with collagen VI, in which NG2 serves as a primary cell surface ligand for collagen VI (Burg et al. 1996). The NG2-Collagen VI interaction has its functional implications: 1) it has been shown that NG2 is very necessary for anchoring Collagen VI to the cell surface (Stallcup et al. 1990; Nishiyama and Stallcup 1993) and 2) this complex interaction enhances the ability of NG2 transfected cells to migrate in response to collagen VI (Burg et al 1997). NG2 is co-localised with type VI collagen in many developing tissues including vasculature and it effectively could anchor collagen type VI to the cell surface (Stallcup et al. 1990; Rand et al. 1993). Experiments performed using NG2 fragments and with NG2 deletion mutants showed that the extended central portion of NG2 (domain 2) is the binding site for collagen VI (Burg et al. 1997; Tillet et al. 1997). It has been shown that the presence of chondroitin sulphate chain is not necessary for NG2 binding to type VI collagen. β -1- integrin is an essential adhesion receptor. However, NG2 ability to bind to type VI collagen does not require the contribution of β - 1 integrin in all cell types. In GD25 cells which are deficient of β 1- subunit and therefore lack the ability to express β 1-containing heterodimers on the cell surface, the presence of NG2 still promotes cell spreading on type VI collagen coated surfaces (Tillet et al. 2002).

NG2 does not interact only with type VI collagen, but it also has the ability to interact with actin cytoskeleton as well. NG2 interaction with the ECM and the actin cytoskeleton, suggests that NG2 could trigger signalling events that lead to the dynamic cytoskeleton

reorganisation and ultimately, affect the cell shape and motility. It has been shown that NG2 can interact with the cytoskeleton (Lin et al. 1996a; Lin et al. 1996b) and found that NG2 stimulation by specific antibodies triggered the signalling events for the melanoma cell spreading (Iida et al. 1995). Furthermore, using cell surfaces that are coated with monoclonal NG2 antibodies as a model, Fang and his colleagues found that the cytoplasmic domain of NG2 appears to be important in NG2 –mediated cell spreading and migration. Elimination of this segment abrogated cell migration (Lin et al. 1996a; Lin et al. 1996b; Fang et al. 1999).

NG2 is co-distributed with the cytoskeleton stress fibres at the cell surface in well spreading cells. It also seems that NG2 use these stress fibres to anchor cells. However, NG2 was also found in actin- positive retraction fibres in rounded or migrating cells (Stallcup 2002). Very early work showed that engagement of NG2 by specific antibodies promoted cell spreading, while anti-NG2 antibodies inhibited attachment, spreading and growth of melanoma cells (Harper and Reisfeld 1987).

There are fourteen potential N-linked glycosylation sites within the extracellular domain of NG2/CSPG4. It is well known that NG2/CSPG4 is first synthesised as a 260kD polypeptide, which is glycosylated to reveal the mature 300kD core glycoprotein (Stallcup et al. 1983). This molecule presents as a proteoglycan molecule and there is evidence that the glycosaminoglycans chain is important for targeting the molecule to a specific micro domain of the cell membrane. The globular domain 3 portion contains N-linked oligosaccharide that is required for galactin-3 and β -1 integrin bindings (Wen et al. 2006). It also contains sites for NG2 proteolysis, leading to release of NG2 from the cell surface (Nishiyama et al. 1991; Asher et al. 2005).

NG2, as other proteoglycans, is subjected to proteolysis. In the previous studies, in 1995, Nishiyama and his colleagues were able to identify three characterised forms of NG2 core protein. The most common form was the core protein of 300kD. The other two forms: 290kD and the 275 forms. The 290kD form was detected in the tissue culture supernatant of B49 cell line (produced from ethylnitrosurea –induced rat brain tumour) and in U251NG52 and U251NG35 cell lines (produced by transfecting U251 MG malignant human glioma cells with the coding region of the rat cDNA NG2). The 290kD form of NG2 lacks the cytoplasmic part but has the entire ectodomain. U251NG52 cell line expressed both the 300kD and 275kD truncated form of NG2. The 275kD form of NG2 lacks the cytoplasmic domain and at 64 amino acids of the ectodomain. The 275 form of NG2 was observed in B49 cell line after mild trypsinization of cells, raising the possibility that this form produced by proteolysis of 300 kD core protein form. Treatment of U251NG52 cell line with Phorbol esters results in the conversion of the 300kD form into 275kD form. Phorbol esters stimulates the activation of protein C kinase, as well as the metalloproteinase such as collagenase and stromelysin, the latter may be responsible for the proteolysis of 300kD from of NG2. B49 cell line did not produce the 275kD form spontaneously; however, the use of NG2 monoclonal antibodies to block the interaction between NG2 and its partner, collagen VI results in the production of the 275kD form. It suggests that the absence of collagen VI renders NG2 more exposed to cell surface proteolysis. Collagen VI contains the knutiz –type proteinase inhibitor sequence in the alpha 3 chain, which may protect NG2 from proteolysis. While the 290kD form usually lacks the entire cytoplasmic domain, but contains the entire ectodomain, the 275kD form lacks the entire cytoplasmic part and at least 64 amino acids from the juxta membranous part. It appears that the 275kD form is truncated at more amino acid sites than the 290 form.

Structural analysis of NG2/CSPG4 showed that its extracellular domain, which contains four internal repeats, possesses a similar sequence to the calcium binding region of cadherins, raising the possibility of involvement of this receptor in calcium- dependent cell signalling interactions (Hatta et al. 1988).

1.4.2The cytoplasmic domain of NG2/CSPG4

The cytoplasmic domain of NG2/CSPG4 has three sites for threonine phosphorylation, but only two of them are sites for functionally important phosphorylation (Nishiyama et al. 1991). Thr-2256 is phosphorylated by PKC α (Makagiansar et al. 2004; Makagiansar et al. 2007) and the Thr-2314 is phosphorylated by ERK (Makagiansar et al. 2004; Makagiansar et al. 2007). This suggests that NG2/CSPG4 may function as a linkage between cells and extracellular matrix (Stallcup et al. 1990; Nishiyama and Stallcup 1993; Burg et al. 1996). Phosphorylation of NG2 at the two threonine sites lying in its cytoplasmic portion has an important role in the cellular behaviour, such as motility and proliferation. This mainly occurs through the ability of NG2 to stimulate the activation of β -1- integrin and determine the localisation of integrin on the cell surface. The ultimate effect will be either to increase cellular motility or proliferation, depending on which site within NG2 is phosphorylated, which will further determine the localisation of β -1 integrin to either the leading edge of the cells or to the micro projections on the apical cell surface (Makagiansar et al. 2007).

In addition, the C terminal half of NG2 is very rich in proline. However, the significance of this remains to be elucidated. There is no significant homology between the cytoplasmic part of NG2 and other proteins, indicating that NG2 is a novel integral membrane protein. At the extreme C-terminus of the cytoplasmic part of NG2, there is a PDZ-binding motif that is

important for the interaction of NG2 with other multi-PDZ scaffolding proteins, MUPP1, GRIP1 and syntenin (Barritt et al. 2000; Stegmuller et al. 2003; Chatterjee et al. 2008)

It is interesting to speculate that extracellular matrix ligands for NG2 might also trigger different types of cytoskeleton rearrangements, depending on which portion of the NG2 ectodomain serves as the site of interaction. Clearly, it will be important for us to determine whether interaction of NG2 with physiological ligands such as type VI collagen results in responses similar to other ECM ligands. There are many possible mechanisms that could explain the possible effects of the interaction of NG2 with actin cytoskeleton or other ECM ligands. The formation of lamellipodia or filopodia occurs with the engagement of NG2 to the ECM substratum; this suggests the involvement of small GTPases (rac and cdc42). However, other adaptor proteins will be required to link NG2 with these signalling pathways. MUPP, a cytoplasmic scaffolding protein, is one of these candidates that bind to the PDZ binding motif of NG2. Other adaptor proteins, such as serotonin and APC tumour suppressor protein are critical for the localisation of NG2 to the sub cellular compartments. Expression of NG2 greatly enhances the formation of retraction formation and the ability of cells to assume the polarized motility characteristic of motile cells.

1.5 NG2/CSPG4 distribution in tissues

1.5.1 NG2/CSPG4 expression in the central nervous system

The expression pattern of NG2 /CSPG4 in the central nervous system was thoroughly studied. It has been shown that NG2/CSPG4 is largely expressed by the oligodendrocytes progenitor cells in the developing tissues of the CNS and by the pericytes associated with the central nervous vasculature. Oligodendrocytes populate the entire system within the CNS and

can differentiate into myelinating oligodendrocytes. NG2/CSPG4 is not expressed by the neural stem cells within the CNS, but is unregulated on the progenitors that emerge from them and differentiate into oligodendrocytes. Once oligodendrocyte progenitors differentiate into myelinating oligodendrocytes, NG2/CSPG4 is down regulated (Nishiyama et al. 1996; Reynolds and Hardy 1997; Trapp et al. 1997; Keirstead et al. 1998; Dawson et al. 2003). NG2/CSPG4 together with PDGFR- α are used as reliable markers for the progenitor cells. PDGFR- α had the same trend of expression as NG2/CSPG4, suggesting that the two molecules are co-ordinately expressed. Even the co-localisation of NG2/CSPG4 and PDGFR- α were reduced as cells enter into the differentiation stage and the correct co-expression of the NG2/CSPG4 proteoglycan and PDGFR- α on the surface of O2A progenitor cells is important for the cells' ability to respond to the mitogenic stimulus of PDGF. This highlights that not only the trend of NG2/CSPG4 expression in tissue is similar to the trend of expression of other molecules but also the functional importance of this similar expression in these cells.

Early on, it had been shown that NG2 positive progenitors are able to mature into both astrocytes and myelinating oligodendrocytes (Stallcup and Beasley 1987; Raff 1989; Horner et al. 2000). Zhu and his colleagues in 2008 found that NG2 positive progenitor oligodendrocytes are able to differentiate into other types of cells within the CNS depending on the cultural environment and this work was further supported by the *in vivo* work. Then NG2/CSPG4 positive cells can give rise to two types of cells. 1) oligodendrocytes in both gray and white matter in the spinal cord and the cerebellum, 2) Astrocytes in the gray matter of the spinal cord but not to astrocytes in the white matter of the spinal cord and the cerebellum (Zhu et al. 2008). This raises not only the importance of NG2/CSPG4 expression in tissues, but also indicates the importance of NG2 in the process of cellular maturation and

differentiation within the CNS. It also demonstrates the developmental plasticity of the NG2/CSPG4 positive cells.

NG2/CSPG4 is also expressed by the tumour cells in the CNS such as gliomas. Glioma is a central nervous system tumour, originates from several cell types, including astrocytes, stem cells and glial progenitors (Singh et al. 2004; Sanai et al. 2005). These cells possess NG2, PDGFR- α and Oligo-2, which are used as markers for these cells (Chekenya et al. 1999; Shoshan et al. 1999; Chekenya and Pilkington 2002; Bouvier et al. 2003). The role of NG2/CSPG4 in glioma progression has been reviewed (Stallcup and Huang 2008). Oligodendrocyte progenitors are the most abundant cells within the adult human brain (Dawson et al. 2000; Mason and Goldman 2002) and it is one of the cell types that glioma originates from. NG2/CSPG4 is one of the markers expressed by the oligodendrocyte progenitors, and it is sensitive to growth factors and extra cellular matrix stimulation, where it potentiates the motility and the proliferation abilities of the cells (Schrappe et al. 1991; Shoshan et al. 1999; Chekenya et al. 2002; Chekenya et al. 2002; Chekenya et al. 2008).. This indicates the potential role of NG2 in increasing the mitogenic activity of these cells and more importantly, in the gliomagenesis process. It has been found that the susceptibility of the progenitor's cells for transformation and formation of gliomas can be explained by their ability to respond to mitogenic stimulus in rodent glioma models (Hesselager et al. 2003; Hu and Holland 2005; Shih and Holland 2006). In gliomas, the level of NG2/CSPG4 expression is correlated with the degree of malignancy. Furthermore, the expression of NG2/CSPG4 is strongly found in the glioma vasculature (Schlingemann et al. 1990; Schrappe et al. 1991; Wesseling et al. 1995; Chekenya et al. 2002). More interestingly, NG2/CSPG4 expression by the glioma cells proved to have an important effect on the vascularity of the gliomas, in

which NG2/CSPG4 provides an important cross talk between tumour-host compartments (Brekke et al. 2006).

Studies done on NG2/CSPG4 expression in different tissues showed that there is a general trend regarding NG2/CSPG4 expression in tissues, where NG2 is not expressed by the multipotent stem cells, while it is up-regulated in cell lineage that is proliferative and developed. However, it is then down-regulated once cells enter into their differentiation stage. More interestingly, it has been found that NG2/CSPG4 is highly expressed in tissue injuries and pathological conditions, including tumours, which indicates that NG2/CSPG4 is regarded as a marker of the activated cells (Stallcup and Huang 2008). For example: in the peripheral nervous system, the mouse NG2 homolog AN2 is reported to be present on Schwann cells (Schneider et al. 2001) and is up-regulated following sciatic nerve injury (Zhang and Ren 2001).

Further studies supported the importance of the presence of NG2/CSPG4 positive cells and its functional implications at both normal and pathological conditions. It has been found that a large number of NG2/CSPG4 positive cells are still present in the spinal cord and the brain of adult rodent, which are either as oligodendrocytes, polydendrocytes or beta neuroglial cells (Dawson et al. 2000; Nishiyama et al. 2002). The importance of the presence of this large number of NG2/CSPG4 positive cells is supported by further studies. It has been found that oligodendrocytes represent, not only a source of remyelination for the demyelinating axons (Gensert and Goldman 1997; Keirstead and Blakemore 1997), but also by increasing the proliferative ability of these cells in response to pathological injuries (Keirstead et al. 1998; Levine and Reynolds 1999; McTigue et al. 2001).

1.5.2NG2/CSPG4 expression outside the central nervous system

Up regulation of NG2/CSPG4 in different developing tissues, which is followed by its down regulation after cellular maturation is the general trend observed in studies performed on different tissues. There is increased evidence that NG2 is up regulated in many types of tissue injuries and pathological conditions, including tumours, which are characterised by increased ability of proliferation and motility as well as by many developing tissues. This indicates that NG2 should not be regarded as a marker for a specific cell type, but it is an indicator for a specific cellular condition, which is an activated versus quiescent status (Stallcup and Huang 2008).

NG2/CSPG4 is expressed by cells derived from the mesenchymal lineage such as chondrocytes. In developing rat limb, staining of the rat limb bud sections showed that NG2/CSPG4 is expressed by pre cartilaginous mesenchymal condensation at embryonic day 14 and up regulated in the differentiating chondroblasts originated from mesenchymal cells by embryonic day 16 in the humerus, femur and digits. However, its expression is down regulated in hypertrophic chondrocytes at day 17 (Nishiyama et al. 1991). These results were confirmed by both immunohistochemistry and northern blotting. They also revealed that NG2/CSPG4 has its distinct features, which is unique from other cartilage proteoglycans. Consistent with this work, NG2 was found to be expressed by the developing cells of the skeletal muscles and bone (Fukushi et al. 2003; Petrini et al. 2003). NG2/CSPG4 is expressed by various tumours, including glioblastoma, melanomas and lymphoid leukaemias (Behm et al. 1996; Shoshan et al. 1999; Lin and Bergles 2002).

There is clear evidence that NG2/CSPG4 is expressed in the vascular structures. NG2/CSPG4 is expressed by the vascular mural cells, and it is not expressed by the endothelial cells lining the blood vessels. In the developing heart, it is expressed by the cardiomyocytes and in the microvasculature; it is present in the pericytes. In the macrovasculature, it is expressed by the smooth muscle cells (Schlingemann et al. 1990; Grako and Stallcup 1995; Burg et al. 1999; Ozerdem et al. 2001; Ozerdem et al. 2002). Added to this, it is known that the pericyte-endothelial relationships are disrupted in the tumour vessels (Morikawa et al. 2002; Bergers and Song 2005; Betsholtz et al. 2005), however, this is more frequently observed in the tumours of NG2 positive "pericyte tubes" segments of blood vessels that are devoid of endothelial cells (Ozerdem and Stallcup 2003). This highlights the importance of the presence of NG2/CSPG4 in the vascular system and its essential role in regulating the pathological conditions associated with it. Furthermore, NG2/CSPG4 is highly expressed in the pericytes associated with wound repair and tumour vasculature (Burg et al. 1999; Morikawa et al. 2002; Ozerdem and Stallcup 2003; Song et al. 2005).

In the skin tissue, NG2/CSPG4 is expressed by the proliferating keratinocytes progenitor cells, which are derived from keratinocytes stem cells that do not show NG2/CSPG4 expression (Legg et al. 2003; Ghali et al. 2004; Kadoya et al. 2008). There is a similar pattern of NG2/CSPG4 expression in the progenitor cells derived from the hair follicle bulge region, where there is a high level of NG2/CSPG4 expression, while NG2/CSPG4 is absent from the mature cells derived from both keratinocytes and bulge region progenitors.

1.5.3 NG2/CSPG4 expression in musculoskeletal tissues

In the bone, Fukushi and his colleagues studied the distribution of NG2/CSPG4 during bone development. It observed that NG2/CSPG4 was strongly up regulated in the immature cartilage, down regulated in mature cartilage and up regulated again during the primary ossification. The same pattern of NG2/CSPG4 expression was seen in the epiphyseal growth plate and in the cranial sutures, where NG2/CSPG4 is highly expressed in the osteoblasts and osteogenic bone fronts respectively, while it is down regulated as the ossification completes (Fukushi et al. 2003). NG2/CSPG4 is expressed by adult human articular cartilage (Midwood and Salter 1998), where its expression is down regulated in osteoarthritic cartilage. NG2/CSPG4 is expressed in chemically induced chondrosarcoma in rat (Leger et al. 1994). However, the expression of this receptor in human chondrosarcoma is not known yet.

1.6 Functions of NG2/CSPG4

The expression pattern of NG2/CSPG4 on the immature progenitor cells described earlier, suggested that NG2/CSPG4 is involved in the process of migration and proliferation, both processes are important for the motility of progenitor cells. This was further confirmed by the finding that NG2 is re-expressed by tumour cells, which are characterised by increased migration and proliferation. Furthermore, NG2/CSPG4 increases the tumorigenic and metastatic properties of mouse melanoma cells (Burg et al. 1998). Subsequent studies were attempted to identify the molecular basis by which NG2/CSPG4 might be involved in different cellular behaviour. It seems that NG2/CSPG4 interaction with extracellular and intracellular partners was the main route by which NG2 could serve as a signalling transduction molecule.

NG2, as other proteoglycans, does not have enzymatic activities and it contains relatively few glycosaminoglycan chains, which all hamper the activity of this receptor to work by its own. Although NG2 does not have the ability to be a signal transducing receptor on its own (Fang et al. 1999; Tillet et al. 2002; Majumdar et al. 2003), it appears that NG2 could potentiate or regulate the activities of more well-known signal transducing system, such as β -1-integrin and growth factors.

1.6.1 The role of NG2/CSPG4 in cell motility

The involvement of NG2/CSPG4 in cell motility was studied and highlighted in different normal and neoplastic cells (Burg et al. 1997; Burg et al. 1998; Grako et al. 1999). It has been shown that NG2/CSPG4's mediating effect on cell motility happens either directly or indirectly, through the cooperative interaction between NG2 and growth factors or beta 1 integrin activation (Fukushi et al. 2004; Makagiansar et al. 2007; Chekenya et al. 2008).

The main point highlighting the involvement of NG2 in cell mobility is by finding that NG2/CSPG4 is a cell surface ligand for collagen VI and this binding has its biochemical and functional implications on different cellular functions (Huang et al.; Stallcup et al. 1990; Nishiyama and Stallcup 1993; Burg et al. 1996; Burg et al. 1997; Tillet et al. 1997). Added to this, the loss of NG2 from the cell surface causes the loss of collagen VI cell surface anchorage, which in turn, reduced collagen VI deposition and poor development and maturation of the tumour blood vessels of NG2 null mice (Huang et al.). Collagen VI binds to the central non globular domain of NG2, which is demonstrated by the solid phase binding assays with purified NG2 fragments and by the recombinant deletion mutants of NG2 in rat B28 glioma cells (Burg et al. 1997; Tillet et al. 1997). B28 glioma cells were transfected with

different mutant cDNA for NG2 and it was found that three mutants (which have deletions in the central one third of the ectodomain) out of 11 were unable to anchor collagen VI. Furthermore, the ability of these three mutants to migrate towards collagen VI was reduced, which indicates the functional importance of this collagen VI-NG2 interaction to the central region of NG2/CSPG4.

In early studies, it has been shown that NG2/CSPG4 can be recognised by specific monoclonal antibodies on the surface of melanoma cells and suggested its involvement in the cell-substrate interactions in the metastatic human melanoma (Bumol et al. 1984; Harper et al. 1984). Later on, it has been shown that NG2/CSPG4 promotes melanoma cell attachment and motility and this effect was inhibited by NG2 antibodies (Burg et al. 1998). In this case, NG2/CSPG4 was able to trigger rearrangement of the actin cytoskeleton (Lin et al. 1996; Lin et al. 1996; Fang et al. 1999). NG2/CSPG4 can trigger the motility process in the cells, even if it is not expressed on their cells surface. It was found that in the vascular endothelial cells, exposure of the cells to NG2 enhanced the cellular motility, promoted endothelial tube formation invitro and increased the blood vessel development *in vivo* (Fukushi et al. 2004). Biochemical data showed the involvement of galectin-3 and alpha3beta1 integrin, where these two molecules form a complex with NG2/CSPG4 on the endothelial cells cell surface. This represents the importance of NG2/CSPG4 as an element of crosstalk between the endothelial and NG2 positive pericytes in the blood vessels. This phenomenon has an important effect on cellular function, especially in tumours conditions.

Later studies were designed to evaluate whether the effect of NG2 on cell motility can occur through a different mechanism whereby NG2 activation may happen through activation of β -

1-integrin. In 2004, Makangiansar and his colleagues discovered a PKC- α -mediated NG2 phosphorylation at the Thr (2256) site, which can initiate the cellular polarization and motility using NG2 transfected U251 cells. In untreated cells, NG2 and $\alpha 3 \beta 1$ integrin are present in apical cell surface protrusions. Phorbol ester treatment causes redistribution of these molecules to lamellipodia, accompanied by increased cell motility (Makagiansar et al. 2004). U251 cells with a valine substitution at the Thr (2256) site did not show any change in the cell motility and NG2/CSPG4 remained at the membrane protrusions, while a glutamic acid substitution at the Thr (2256) site rendered cells spontaneously motile even without phorbol ester treatment and re-distribution of NG2 to the lamellipodia occurred.

Makagiansar and his colleagues as well in 2007 showed that NG2/CSPG4 has two phosphorylation sites and their activation can have a differential effect on cellular functions. When NG2/CSPG4 is phosphorylated at the Thr (2256) site, NG2 co-localizes with $\beta 1$ -integrin on lamellipodia at the leading edges of cells, leading to enhanced cell motility. Whereas phosphorylation of NG2/CSPG4 at the Thr2314 site results in its co localisation with $\beta 1$ -integrin on the micro protrusions from the apical cell surface, which in turn promotes cell proliferation. Therefore, the phosphorylation of NG2/CCSPG4 causes the re-localisation of NG2 and its associated molecule, $\beta 1$ - integrin to different cellular parts, which is very important in determining the differential cellular response to either increase the cell motility or cell proliferation (Makagiansar et al. 2007).

NG2/CSPG4 can increase cell motility through its interaction with growth factors, such as PDGF-AA. In aortic smooth muscle cells, NG2 null cells were unable to proliferate or migrate normally in response to PDGF-AA, due to poor activation of PDGFR- α , while wild type cells exhibited very good proliferation and migration abilities in response to this factor (Ozerdem and Stallcup 2004).

1.6.2 The role of NG2/CSPG4 in cell survival and chemoresistance

Chemoresistance is one of the challenges facing clinician in the treatment of cancers; one of them is chondrosarcoma, which is a well known chemo-resistant tumour (Jamil et al. 2010). Chemotherapeutic drugs induce apoptosis in cancer cells. Several studies have highlighted the importance of NG2 in tumour progression, through its effect on cell invasion, angiogenesis, apoptosis, and necrosis. The role of NG2/CSPG4 in regulating cell death has begun to be explored. Wang and his colleagues (2011) found that targeting NG2/CSPG4 reduces the apoptosis and necrosis rates in xenografts of glioblastomas cell lines and A375 melanoma tumours indicating that NG2/CSPG4 affects the tumour pathology through multiple mechanisms (Wang et al. 2011).

NG2/CSPG4 over expression has been reported in some chemo-resistant tumours, including glioblastomas, most melanomas and some leukaemia (Schrappé et al. 1991; Behm et al. 1996; Mauvieux et al. 1999; Shoshan et al. 1999; Chekenya et al. 2002; Li et al. 2003). In glioblastomas, NG2-transfected U251glioma cells were resistant to cytotoxic drugs (Doxorubicin, Vincristine and Etoposide) and TNF- α through increased activity of $\alpha 3\beta 1$ integrin/ PI3K signalling and their downstream targets, which all promote cell survival (Joy et al. 2003; Downward 2004; Chekenya et al. 2008). siRNA mediated NG2/CSPG4

knockdown sensitized the NG2-transfected U251 glioma cells to cytotoxic treatment, which further supports the previous data and highlights the relationship between NG2 and apoptosis resistance. In the same study, it has been found that NG2 knockdown was effective in establishing the apoptosis sensitivity in endogenous NG2 expressing glioma lines such as U87 and A172, as well as in the A375 melanoma line, which suggests that NG2/CSPG4 may be an effective therapeutic target in several cancer subtypes (Chatterjee et al. 2008). The role of NG2 dependent apoptosis mechanism in this study was further supported by *in vivo* work, whereby the presence of NG2 enhances the tumour growth, promotes the chemoresistance to cytotoxic drugs and demonstrates a strong correlation between the level of NG2 expression and apoptosis resistance in tumour samples.

On the other hand, NG2/CSPG4 can serve as a proapoptotic receptor. This was demonstrated in fibroblasts by Joo and his colleagues (2008). It is known that altered fibronectin (FN) causes a disruption of cell–matrix interactions and leads to apoptosis. Reduced FAK phosphorylation is important for this process (Kapila et al. 1999; Kapila et al. 2002; Tafolla et al. 2005). It is known that NG2 regulates FAK phosphorylation through PKC- α in human melanoma cells and NG2 and $\alpha 4\beta 1$ integrins acts as co receptors mediating the spreading of melanoma cells on FN coated surfaces (Iida et al. 1995; Yang et al. 2004). In this study, altered FN caused a reduced phosphorylation of FAK, up regulation of NG2 and down regulation of integrin $\alpha 4$. The role of NG2/CSPG4 in this study was opposite to integrins in regulating anoikis and NG2 and integrin $\alpha 4$ regulate FAK phosphorylation by PKC- α dependent and -independent pathways respectively. The role of NG2/CSPG4 in regulating cell death may be important in human chondrosarcoma. This might lead to further studies in this aspect.

1.6.3 The role of NG2/CSPG4 in cell proliferation

The role of NG2/CSPG4 in cell proliferation was studied in detail in both normal and diseased conditions. The idea of involvement of NG2 in cell proliferation was supported by the fact that NG2/CSPG4 is capable of interacting with high affinity to the growth factors: basic fibroblast growth factor (bFGF, also known as FGF-2) and platelet derived growth factor alpha (PDGF-AA). NG2/CSPG4 interacts with the growth factors through its core protein part rather than the chondroitin sulphate chain (Goretzki et al. 1999).

FGF-2, a well-known member of the FGF family, was originally isolated and identified based on its stimulatory activity on fibroblast proliferation (Lobb et al. 1986). Its functional roles are extensively studied and shown to be involved in numerous cellular functions in various cell types, including angiogenesis, tumorigenesis, cell proliferation, differentiation, wound healing, limb formation, and tissue remodelling (Bodo et al. 2002; Bobick et al. 2007; Douwes Dekker et al. 2007; Kakudo et al. 2007; Kanayama et al. 2007; Pratsinis and Kletsas 2007; Schmal et al. 2007 and Choi et al. 2008). It has been found that FGF-2 plays an essential role in cartilage matrix homeostasis. FGF2 is produced by chondrocytes, stored in the ECM, and immediately released from the ECM upon cartilage injury (Vincent et al. 2002; Vincent et al. 2004). FGF-2 has a potent mitogenic impact on the growth plate of the cartilage (Rosselot et al. 1994) and adult articular cartilage (Osborn et al. 1989; Stewart et al. 2007).

Platelet-derived growth factors (PDGFs) are main mitogens for many cell types e.g. fibroblasts and smooth muscle cells. The PDGFs and their tyrosine kinase receptors, PDGF receptor - α and PDGF receptor β , are one of the main growth factors- growth factors systems that are studied in depth. All PDGFs contain: growth factor domain, N- and C-

terminal polypeptide sequences that are involved in the regulation of the biological activity of these factors, and determines their ability to interact with the extracellular matrix. There are at least 2 binding sites for PDGF-AA and FGF-2 within the ectodomain part of NG2/CSPG4. NG2/CSPG4 appears to help these growth factors, by sequestering them at the cell surface and presenting them to their respective receptors (Stallcup 2002).

An initial study carried out on O2A oligodendrocytes progenitor cells showed that treatment of these cells with NG2 blocking antibody *in vitro* inhibited the proliferation of these cells. In addition, there was a reduced expression of PDGF alpha- receptors in these cells (Nishiyama et al. 1996). Therefore, this highlights the importance of the co-expression of the NG2 proteoglycans and PDGF receptor- α on the surface of O2A progenitor cells as this can determine the ability of these cells to respond effectively to the mitogenic stimulus of PDGF.

This was further supported by another research study, showing that NG2 NG2-null progenitor cells (A2B5+O4-) derived from NG2 knockout mice were unable to maintain their proliferative status in response to bFGF and PDGF-AA, in comparison to the wild type (A2B5+O4-) cells (Grako and Stallcup 1995). Over a course of several days, NG2 NG2-null progenitors could not maintain their undifferentiated state, did not respond to growth factors and progressed to A2B5-O4+ phenotype characteristic of initial differentiation along the oligodendrocyte pathway.

In aortic smooth muscle cells, NG2 null cells were unable to proliferate or migrate normally in response to PDGF-AA, due to poor activation of PDGF receptor- α , while wild type cells exhibited a very good proliferation and migration abilities in response to this factor.

However, both cell types maintained their mitogenic and proliferative properties in response to PDGF-BB (Grako et al. 1999). There was no autophosphorylation of PDGF receptor- α in treated null cells which highlighted that a defective signal transduction happened at the PDGF- α receptors after NG2 loss from these cells. It also indicated that the activation of the extracellular signal-regulated kinase (ERK) in response to PDGF-AA and PDGF-BB occurred at different levels and appeared that NG2 loss affects the mitogenic response to PDGF-AA rather than PDGF-BB.

There are unpublished data collated from research in the laboratory of Dr. Roberto Perris and referred to in Stallcup and Huang review paper in 2008, explained that the lack of NG2 from the smooth muscle cells makes them less responsive to FGF-2, resulting in reduced activation of FGF-2 receptor and consequently low proliferative response. This was more or less similar to the idea obtained by Ozerdem and Stallcup in their published paper in 2004, which showed that cells lacking NG2 failed to sequester FGF-2 growth factor, resulting in reduced activation of FGF-2 receptors as well as a reduction in the FGF-2 mitogenic activity. *In vivo* work using the corneal angiogenesis model in which an FGF2-containing pellet was used to stimulate blood vessel growth into the corneas of wild type and NG2 null mice further supported the previous data. Cellular response to FGF2 was excellent in wild type corneas, but reduced by at least four-fold in NG2 null corneas. This mainly occurred as a result of poor pericyte proliferation in the absence of NG2. Using BrdU incorporation to label mitotic cells showed that the proliferation of NG2 null pericytes was reduced by a factor of three compared to wild type pericytes, presumably due to lack of responsiveness to FGF2 (Ozerdem and Stallcup 2004).

Literature studies showed that the phosphorylation of NG2 at the Thr-2314 by PKC- α caused an increase in cell proliferation and even at the basal level without cellular stimulation, NG2 transfected U251 glioma cells are more proliferative than parental U251 cells due to a higher level of phosphorylated NG2 in these cells (Makagiansar et al 2007). It has been found that phosphorylation of NG2 at its two distinct phosphorylation sites (Thr-2256 and Thr-2314) helps to balance the two cellular functions (motility and proliferation respectively). B-1-integrin activation appears to have an important role in this NG2- dependent cell proliferation or motility mechanisms (Makagiansar et al. 2004; Makagiansar et al. 2007).

1.6.4 The role of NG2/CSPG4 in angiogenesis

It is well known that both endothelial cells as well as the pericytes are involved in the development of the micro vascular system. In some systems, pericytes are among the first cell types involved in nascent blood vessels, sometimes even preceding the involvement of endothelial cells (Nehls et al. 1992; Wesseling et al. 1995; Redmer et al. 2001; Ozerdem and Stallcup 2003; Tigges et al. 2008). NG2 has its' effects on both endothelial and pericyte components of the micro vascular development.

For the role of NG2 in the pericyte development and function, postnatal neovascularisation was reduced in NG2 null mouse. In ischemic retinal vascularisation and in corneal vascularisation induced by FGF2, blood vessel development is decreased more than 2-fold by the knockdown of NG2 (Ozerdem and Stallcup 2004) due to diminished pericytes: endothelial cell ratio (dropping from 1:1 to as low as 1:4) in NG2-deficient vasculature of these eye models. The reduced proliferation of pericyte in the absence of NG2 (detected by BrdU incorporation) was the main possible reason for this effect.

The effect of NG2/CSPG4 knockdown on endothelial cells has also been addressed. A reduction in the endothelial cells proliferation was also observed, but to a lesser extent than that of pericytes. Unpublished data, but mentioned in Stallcup and Huang review paper (2008)(Chekenya et al. 2008), indicated that, when NG2 is knocked down, mammary tumours in the MMTV-PyMT transgenic mouse model develop more slowly and with reduced metastasis (Webster 1998; Maglione et al. 2001). Although NG2 is not expressed by the mammary tumour cells in this model, it appears that NG2 has an effect on parts of the tumour stroma. So vessels in NG2 null tumours exhibit reduced diameters and diminished investment by pericytes compared to the blood vessels in wild type tumours,

Recently, a study done in 2011 by Chekenya and her colleagues found that targeting NG2/CSPG4 reduces the growth rate of the tumours, angiogenesis and vascular permeability, in xenografts of glioblastomas cell lines and A375 melanoma tumours. All this work highlights the importance of NG2/CSPG4 as an anti-angiogenic therapy in the treatment of cancer (Wang et al.).

1.6.5 The role of NG2/CSPG4 in human articular cartilage

A role for NG2/CSPG4 in osteoarthritis was explored (Midwood and Salter 1998). It has been reported that NG2/CSPG4 modulates the interaction of normal articular chondrocytes with type VI collagen indirectly and this interaction has been lost in osteoarthritis, suggesting that these receptors can affect the progression of this disease (Midwood and Salter 2001). It is however unclear what roles NG2/CSPG4 may have in OA. The apoptotic death of articular chondrocytes has been implicated in the pathogenesis of osteoarthritis and the role of NG2/CSPG4 in modulating apoptotic cell deaths started to be explored. The clinical

challenge now is to explore a treatment for metastatic and unresectable chondrosarcomas. Therefore, it is possible that NG2 might be involved in the pathogenesis of OA and can serve as a therapeutic target in both OA and human chondrosarcoma.

1.7 NG2/CSPG4 shedding and its functional implications

Many Integral membrane proteins exist in two forms: membrane bound and soluble forms. There are 2 possible mechanisms for the production of the truncated and soluble forms of integral membrane proteins: 1) can arise from a separate gene by multiple splice variant or 2) as a result of post- translational modifications of the intact protein. Attempts have been made to define the functional properties of these forms, mainly through their ability to interact with the growth factor receptor molecules. For example, in Schwann cells, the truncated form of low affinity growth factor receptor released by the metalloproteinase cleavage is involved in the regulation of nerve regeneration (DiStefano et al. 1993).

The possibility that different forms of NG2 could arise at the transcriptional level by an alternative splice variant has been tested in 1995 by Nishiyama and his colleagues. They found that, in B49 rat cell lines (U251NG52 and 53), NG2 core protein is expressed as three forms (300, 290 and 275kD). These forms originated from the same splice variant. However, the 300 core protein had undergone a proteolytic cleavage to yield the 275- and the 290-kDa forms. This point was further studied in this article and the activation of the protein kinase C stimulated the conversion of the 300 core protein form. Blocking the interaction between NG2 and collagen type VI was found to protect the NG2 against cell surface proteolysis. Protein kinase C activity was important to determine the relative proportion of 275 and 300kD proteins detected. When a pulse-chase experiment was performed, it was predicted

that protein kinase C induced a transcription of gene encoding a proteinase that was responsible for the cleavage of NG2. Although the exact enzyme and the site for NG2 cleavage was not clarified, metalloproteinases, such as collagenase and stromelysin, may have a role in this process, as NG2/CSPG4 contains cleavage sites for these two metalloproteinases. The authors hypothesised that the 275kD form was not released from the cell surface because of a non covalent protein-protein interaction occurring between two sides on either sides of the cleavage. But this effectively reconnects the large N-terminal domain with the cell associated C-terminal stub (Nishiyama et al. 1995).

NG2 is susceptible to a separate (non shedding) and shedding metalloproteinase-mediated effect, where the use of metalloproteinase inhibitors were able to prevent NG2 shedding in the oligodendrocytes progenitors, while the generation of truncated form of NG2 was sensitive to TIMP-2 and TIMP-3 *in vivo* (Asher et al. 2005). It was not clear which metalloproteinase was really involved in NG2 shedding in ODP, however, more supportive data were in favour of the role of ADAMT 8 in this process.

A study done in 2003 showed that, in both rat and human peripheral nerves, all NG2 is saline soluble(shed), which represents the shed ectodomain NG2 (Morgenstern et al. 2003). Then a later research study carried out by Asher et al in 2005 showed that a high proportion of NG2 in adult rat spinal cord is saline soluble (shed) and over expression of NG2 in injured cerebral cortex is mainly due to saline soluble (shed) rather than detergent-soluble (intact) form. This indicates two things: 1) the rate of NG2 shedding is greater in the CNS injury and 2) the presence of high proportion of NG2 shedding may have its' roles in axon regeneration in the CNS. The saline soluble NG2 migrated faster on SDS gel rather than the detergent one,

indicating that the saline soluble form of NG2 represented the shed ectodomain of NG2. It is well known that NG2 exerts an inhibitory effect on the axon growth in the CNS (Ughrin et al 2003). However, it seems to be that the high percentage of NG2 shedding would have a more pronounced effect in this case.

1.8 Hypothesis

NG2/CSPG4 is an important regulator of chondrocyte function and has the potential to be a therapeutic target for treatment of diseases of cartilage, such as osteoarthritis and chondrosarcoma.

1.9 Aims and objectives

1. To confirm that NG2/CSPG4 receptor is expressed in human chondrosarcoma.
2. To investigate and compare NG2/CSPG4 expression, biochemical properties and localisation in osteoarthritic human articular chondrocytes and transformed and chondrosarcoma cell lines.
3. To develop an *in vitro* model to assess NG2/CSPG4 function (NG2/CSPG4 knock-down in JJ012 cell line).
4. To investigate the functional roles of NG2/CSPG4 in human chondrocytes in :
 - Cell adhesion and spreading.
 - Cell migration and invasion
 - Cell proliferation.
 - Apoptotic cell death and chemoresistance.
5. To demonstrate the effect of NG2/CSPG4 loss on cartilage phenotypes and genes involved in the pathogenesis of OA process.

CHAPTER TWO

Materials and Methods

All reagents and tissue culture media were supplied by Sigma, UK unless otherwise stated.

Details of all antibodies used in this study are summarised in tables (2.5 and 2.6), details for all primers used are in table (2.7) at the end of this chapter.

2.1 NG2/CSPG4 expression *in vivo*

2.1.1 Immunohistochemistry

Materials used in this procedure were supplied by DAKO, UK. Slides were fixed in acetone for 2 minutes, loaded into the Sequenza racks (Shandon, UK) and washed with PBS. Slides were incubated with hydrogen peroxide for 5 minutes, washed and incubated with 100µl of NG2 (LHM2) antibody overnight in the cold room. Next day, the slides were washed, incubated with 100µl of Envision reagent for 30 minutes and washed with distilled water for 5 minutes. 0.5ml of DAB (made up with 11 drops of DAB Chromogen in 11ml of DAB Buffered Substrate) was added 3x 5 minutes. Slides were washed with distilled water for 5 minutes, tap water for 5 minutes, stained by placing in copper sulphate solution for 3 minutes, washed again and counterstained in Haematoxylin for 1 minute, differentiated in acid alcohol, and 'blued up' by placing in Scott's tap water substitute. Slides were dehydrated by passing through ascending alcohol concentrations, cleared in xylene and mounted in Pertex.

2.1.2 RNA extraction from frozen sections of the cartilage

RNA was extracted from the frozen sections of normal and osteoarthritic cartilage following Qiagen method. Cartilage sections were cut into (5–10µm) thick and 80 sections was used/

sample/tube. 1ml of QIAzol lysis reagent (Qiagen, UK) was added to sections of the frozen cartilage and incubated at room temperature for 5 minutes. Then, 0.2ml chloroform per 1ml QIAzol lysis reagent was added to the sample, the lid securely closed the lid containing the homogenate, and shaken vigorously for 15 seconds. Then the sample was placed on the bench top at room temperature for 3 minutes and then centrifuged at 12,000 x g for 15 minutes at 4°C. After centrifugation, the sample's content was separated into 3 phases: an upper, colourless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. The upper, aqueous phase was transferred into a new tube by pipetting.

0.5ml isopropanol per 1ml Qiazol lysis reagent was added to the tube, mixed thoroughly by vortexing and incubated at room temperature for 10minutes. Then samples were centrifuged at 12,000 x g for 10 minutes at 4°C and the supernatant was discarded. The RNA pellet was often visible as a gel-like or white pellet at the bottom of the tube. 1ml of 75% ethanol per 1ml QIAzol lysis reagent was added to the tube and centrifuged at 7500 x g for 5 minutes at 4°C. If the RNA pellet floats or sticks to the side of the tube, it should be brought to the bottom of the tube by centrifuging at 12,000 x g for 5 minutes at 4°C. The supernatant was removed completely, and the RNA pellet was dried off by incubating it at room temperature with the lid open. The RNA pellet was dissolved into 30µls of RNase-free water. After which, RNA was cleaned up using RNeasy Mini Elute columns. The RNA was centrifuged at 13000 revolutions per minutes (rpm) xg for 5 minutes, the column was discarded and the RNA was collected from the bottom of the tube. RNA was stored in the freezer at -20°C to be used later.

2.1.3 Real time PCR

The reaction was carried out using the SYBR Green method (Qiagen, UK). 2x Quanti Tect SYBR Green PCR Master Mix (stored at -20°C) was thawed properly at RT, together with

the cDNA, primers, and RNase-free water. All the reaction's materials were kept on ice throughout the reaction. The reaction was made up in 96 well optical plates (AB Bio system ,UK); and mixed according to the table shown below. The negative control (no cDNA added and replaced with water) was included. It was necessary to keep samples on ice during reaction setup and while programming the real-time cycler (Applied Biosystems 7500 thermal cycler). Following this, the reaction was put in the real time cycler machine and the relevant PCR programme was selected (details shown below). Forty cycles were used for all the primers used in this study. Data acquisition was performed during the extension step. The melting curve analysis was performed before using any new primer.

Table 2.1: The table below shows the different reagents used to prepare the master mix.

Reagent	Volume (n) NG2	Volume (n) GAPDH, CO1 or B2M
2x Quanti Tect SYBR Green	12.5 µl	12.5 µl
Forward primer	0.6µl	0.3µl
Reverse primer	0.3µl	0.3µl
RNAse free water	9.6µl	9.9µl
cDNA	2µl	2µl
Total volume	25µl	25µl

2.2NG2/CSPG4 expression *in vitro*

2.2.1 JJ012, chondrosarcoma cell line, culture

The JJ012 (grade II chondrosarcoma) cell line was kindly donated by Professor Joel A. Block, Rush University Medical Centre, Chicago, USA. JJ012 medium (500ml) was prepared from: 200ml of DMEM (40%), 50ml of F12 (10%) (Gibco, UK), 200ml of MEM α (40%) (Gibco, UK), 50ml of FBS (10%), 5ml of penicillin/streptomycin (1 μ l/ml), 1.2 μ l of insulin, 500 μ l ascorbic acid, 562 μ l hydrocortisone.

2.2.2 Resuscitation of JJ012 cells

An aliquot of cells were removed from liquid nitrogen, mixed with 10ml medium and centrifuged at 177 RCF for 5 minutes at 4°C. The supernatant was discarded and cells were then resuspended in 10ml medium and seeded in 75 cm² flask. Cells were grown in the incubator at 37°C with 5% CO₂.

2.2.3 Passaging of JJ012 cell line

1ml of EDTA/ trypsin was added to cells and incubated for 5 minutes Cells were washed; cell suspension was collected and centrifuged for 5 minutes at 707 RCF. Cells were re-suspended in medium and passaged when confluent (2-3 times/ week).

2.2.4 Freezing down of JJ012 cell line

Freezing medium was prepared from (10% DMSO, 60% FCS and 30% DMEM). Cells were passaged. Five-ten x 10⁶ cells/ml were added/ vial. Cells were stored in -80 freezer for one day before being moved to the liquid nitrogen storage area for long term storage.

2.2.5 Primary human articular chondrocytes culture

2.2.5.1 Tissue sources and handling

Human articular tissues were obtained from human cartilage, either from post mortem examination (normal), or from human adult's knee joints undergoing knee replacement surgery (arthroplasty) (OA), with informed consent from Edinburgh Royal Infirmary. Tissues were processed in a class II tissue culture hood.

2.2.5.2 Assessment of the cartilage for osteoarthritis (OA)

Articular surface was assessed macroscopically for the presence or absence of osteoarthritis according to Collins and McElligott Grading system (Collin McElligott 1960; Midwod and Salter 1998) (Table 2.2). Two pieces of cartilage were taken from normal or OA cartilage for histological confirmation by using a scalpel from a representative area of the cartilage. One piece was snap frozen in liquid nitrogen and stored in cryovials at -80 °C for frozen sections. Another piece was fixed in 4% formalin for embedding in paraffin wax.

Table 2.2: Collins macroscopic grading for osteoarthritis (OA)

Collins McElligott Grading	Description
0	No cartilage degeneration (normal cartilage)
I	Patches of fibrillation and softening in central areas.
II	Fibrillation more pronounced and early osteophytosis.
III	Commencing exposure of subchondral bone (osteophytes).
IV	Extensive cartilage loss and bone exposure.

2.5.3 Isolation of primary human articular chondrocytes from human cartilage

Pieces of cartilage were removed from the knee joints, using sterile forceps and scalpel, placed into a petri dish, containing antimicrobial solution (see appendix I). These large pieces (around 4-5mm size) were chopped into small pieces, around (1-2) mm, and left to soak in antimicrobial solution for an hour at room temperature. The antimicrobial solution was removed with pastette and cartilage pieces were washed with phosphate buffered saline (PBS) twice. 20ml of Trypsin enzyme (1X) was added to digest cartilage pieces and left for 30 minutes at 37°C in 5% CO₂ in the tissue culture incubator. Trypsin was removed with pastette and cartilage pieces were washed once in PBS. This was followed by the addition of 60U collagenase Blend H (see appendix I) and left to digest at 37°C in 5% CO₂ in the incubator overnight.

Cell suspension was collected with sterile pastette, strained through a sterile metal strainer into a 50ml centrifuge tube and cell pellets were obtained by centrifuging at 2000 rpm for 5 minutes. The supernatant was discarded and cell pellets were washed with sterile PBS. Centrifugation was performed three times with the disruption of cell pellets each time to allow proper removal of any debris. Cells were counted using a haemocytometer. Cell viability was assessed and should be not less than 95%. Cells were seeded at a density of 5×10^4 /ml in 58mm Petri dish (Nunc, UK) in monolayer cell culture for protein and RNA extraction. Cells were cultured in seeding media (appendix I), in a humidified incubator at 37°C and 5% CO₂, for an average of 4-5 days. Then the media was changed to feeding media (appendix I) and cells were cultured for up to 15-20 days, feeding twice per week until 80 % confluent in dishes.

For indirect immunofluorescence, chondrocytes from OA adult articular cartilage were cultured at a concentration of 5×10^4 /ml as a monolayer for 10 to 15 days in a 75 cm² flasks. Next, cells were detached using 5ml of Trypsin/ EDTA (1X). Cells were washed with PBS and cultured in chamber slides (Lab-Tek, UK), at cell density of 4×10^4 /400µl for 3-4 days until they became 60-70% confluent.

2.2.6 Chondrocyte cell line culture

The chondrocyte cell line, C₂₀A₄ was used. An aliquot was taken from liquid nitrogen and gently shaken in a 37°C water bath for 4-5 minutes. Then cells were put immediately into a tube and centrifuged for 5 minutes at 2000 rpm xg. The supernatant was removed and cells were mixed with Dulbecco's Modified Eagle's Media (DMEM) (see appendix I). Cells were cultured into a 75 cm² flask at 37°C with 5% CO₂, until approximately 80% confluent.

Cells were then passaged once a week and fed every 3 days. To passage C₂₀A₄ cells, media was removed, the flask was washed with 20ml PBS and 0.5ml of Trypsin/ EDTA (1X) (Sigma, UK) was added to the flask. Cells were incubated at 37°C, 5% CO₂ for 5 minutes in the tissue culture room in the incubator. The flask was shaken gently to detach cells and the cells washed with 30ml PBS. The solution was collected and centrifuged for 5 minutes at 2000 rpm. The supernatant was discarded and cells were washed with 30ml PBS once and centrifuged again. Cells were resuspended in 5ml DMEM media and counted using a Haemocytometer. To maintain cells in culture while preserving their phenotypic characters, cells were then passaged once a week and fed every 3 days.

To passage C₂₀A₄, media was removed, the flask was washed with 20ml PBS and 0.5ml of trypsin/ EDTA (1X) was added to the flask. Cells were incubated at 37°C, 5% CO₂ for 5

minutes in tissue culture room incubator. The flask was shaken gently to detach cells and the cells washed with 30ml PBS. The solution was collected and centrifuged for 5 minutes at 2000 rpm xg. The supernatant was discarded and cells were washed with 30ml PBS once and centrifuged again. Cells were resuspended in 5ml DMEM media and counted using a haemocytometer. For indirect immunofluorescence cells were seeded in chamber slides at a cell density of $2 \times 10^4/400\mu\text{l}$ s and grown in culture for 2-3days until they became 60-70% confluent.

2.2.7 Gene expression

2.2.7.1 RNA extraction

The RNase Mini Kit from Qiagen (UK) was used. The extraction was performed following the manufacturer's instructions. Cells were washed with PBS, 350 μl RLT buffer was added and cells were detached. The cell suspension was collected. An equal volume of 70% sterile ethanol was added. 350 μl of RW1 were added and ultra centrifuged for 1 minute at 13000 rpm. 10 μl DNase stock solution was mixed with 70 μl Buffer RDD (Qiagen, UK) and incubated at RT for 15 minutes 350 μl of RW1 and was then added and centrifuged. 500 μl of RPE buffer was added and ultra centrifuged at 13,000 rpm xg for 2 minutes. Spin columns were removed and placed in 1.5ml RNase free tubes. 30 μl of RNase free water was added before centrifugation to elute RNA.

2.2.7.2 RNA quantification using the Nanodrop spectrophotometer

RNA quantification was carried out using the Nanodrop1000 spectrophotometer (Thermo Scientific, UK). 1.5µl RNase free water was used to blank the machine and then samples measured.

2.2.7.3 Test RNA integrity and check for genomic DNA contamination

To check integrity of RNA, extracts were run in a 1.5% agarose gel. 3µl RNA was mixed with the same amount of 2X orange G loading dye. The gel was run for 35 minutes at 70V and developed using the Versadoc machine. To check for genomic DNA contamination, the RNA integrity test was used, but cDNA was added to reactions instead of RNA. Samples were then run as above.

2.2.7.4 Reverse Transcription

This procedure was carried out using Qiagen Long Range 2 step RT- PCR kit. Mastermix was prepared (see appendix I). Reactions were prepared by mixing 8.2µl mastermix and 0.5µg RNA and RNase free water to make up to 20µl. Samples were placed in (G-Storm) thermal cycler for 90 minutes at 42 °C followed by 5 minutes at 58 °C.

2.2.7.5 Polymerase chain reaction (PCR)

Mastermix was prepared (appendix I), 18µl master mix was mixed with 2µl cDNA.

2.2.7.6 Agarose gel electrophoresis

2% agarose gel was prepared (see appendix I) in a conical flask and melted in a microwave. The gel was allowed to cool down slightly, SYBR safe stain (Invitrogen, UK) was added (1/10,000 dilution), and poured into a gel tray. The gel was left to set at RT. Once the gel was

solid, the gel and the tray were put in the tank (H3-Set, Anachem), and covered with TBE buffer (see appendix I). Samples were mixed with the loading dye5x. Samples were loaded on gel. The gel run at 100 V for 50 minutes and analysed using the Versadoc machine (Bio Rad, UK).

2.2.8 Western blotting

2.2.8.1 Protein extraction

Three different buffers were used for protein extraction Sodium orthovanadate, RIPA Buffer and SDS lysis buffer (see appendix for preparation details of all buffers used). Cells were washed with 5ml wash buffer on ice; 350µl of lysis buffer was added, incubated for 15 minutes and lysats collected. Cell lysates were boiled for 10 minutes when SDS used. Lysates were ultracentrifuged for 10 minutes at 13000 rpm xg at 4°C.

NG2/CSPG4 is a trans-membrane proteoglycan receptor expressed as a high molecular weight protein >400kD (chondroitin sulphated form) and a core protein of 270-300kD (chondroitin sulphate free). Treatment of cells with chondroitinase ABC deglycosylates NG2/CSPG4 results in the expression of chondroitin sulphate free core protein. For chondroitinase ABC treatment, cells were extracted and incubated with chondroitinase ABC at 1U/ml for 45 minutes at RT before centrifugation.

2.2.8.2 BCA assay determination of protein concentration

Pierce BCA kit (Thermo Scientific, UK) was used. BSA standards were prepared from PBS and bovine serum albumin (BSA) (appendix I) and added to 96 well plates in triplicate. Test samples were prepared by mixing 2.5µl protein extract with 22.5µls PBS and added in

triplicate. The working reagent was prepared by mixing 10ml of solution A and 0.2ml of solution B (provided in kit). 250µl of working reagent was added to each well and incubated for 30 minutes at 37°C. The plate was read using BioTek spectrophotometer at 562 nm.

2.2.8.3 Sample preparation and running procedure

All reagents and materials used in sample preparation, running and blotting step came from Invitrogen (UK). The method was performed according to the manufacturer's instructions. 5µl loading buffer, 2µl reducing reagent and 13µls sample were mixed and put in a thermal cycler at 70°C for 10 minutes. 2-8% tris-acetate gradient gel was used. The middle of the western blotting tank was filled with running buffer mixed with 200µl antioxidant and the rest of the tank was filled with running buffer only. 20µl of samples were loaded. Gel was run at 200 V for 35 minutes

2.2.8.4 Blotting step

The transfer buffer was prepared (appendix I). PVDF (BDH, UK) membrane was presoaked in 100% methanol for 1-2 minutes and then in distilled water for 1-2 minutes. The membrane was put in transfer buffer. Filter papers and sponges were presoaked in transfer buffer. The orientation of layers in the blot module was as follows: sponge pad, filter paper gel, membrane, filter paper, sponge pad. The outer part of the tank was filled with Milli Q water. The gel was run at 30 V for an hour (1 gel) or 1.5 hours (2 gels).

2.2.8.5 Blocking step

The membrane was incubated with 20ml of 5% Marvel for 2 hours. Membranes were washed with TBST for 3x5minutes and incubated with primary antibody overnight at 4°C. The antibodies (NG2 antibodies and α - tubulin) were diluted in 5% Marvel. The membrane

was washed again and incubated with secondary antibody for 1 hour at RT. The membrane was washed, incubated with ECL plus (Amersham, UK) and visualised using the Versadoc machine.

2.2.9 Indirect Immunofluorescence

Cells were washed with PBS and fixed with 1:1 acetone/methanol solution for 5 minutes at – 20°C. Nonspecific background was blocked with 10% goat serum for 1 hour at RT. Cells were washed with 5ml PBS 2x5 minutes, and incubated with 300µls of NG2 (LHM2) antibody in 1% goat serum for 1 hour at RT.

Cells were washed and incubated with 300µls AlexaFluor 488 goat anti-mouse secondary antibody for 1 hour at RT in the dark. Cells were stained with 300µls propidium iodide, for 5 minutes and washed in PBS. Dako fluorescent mounting media was used and images were visualised using the confocal microscope.

2.2.10 Double Immunofluorescence for NG2/CSPG4 co-localisation with ER, Golgi complex

The media was poured off and cells were washed once in PBS. Cells were fixed with 2ml of 4% paraformaldehyde at room temperature for 10 minutes. Paraformaldehyde was removed from dishes and cells were allowed to air dry for 15 minutes. Non-specific binding was blocked with 10% goat serum for 1 hour at RT using 300µls/dish. Cells were washed with PBS, 2 x 5 minutes. Then cells were incubated with primary antibody or antibodies (details shown in table 2.3), diluted in 1% goat serum, for 1 hour at RT. Cells were washed again with PBS, 2 x 5 minutes. Cells were incubated with secondary antibody or antibodies (details shown in table 2.3), diluted in 1% goat serum, for 1 hour at RT and covered with tin foil.

Cells were washed once with PBS for 5 minutes and stained with DAPI (1mg/ml) diluted in PBS for 5 minutes. Cells were washed once with PBS for 5 minutes. Slides were mounted in Dako fluorescent mounting media and the excess media was removed. Slides were stored at 4°C and covered in tin foil.

Table 2.3: The table below shows the details of all dishes included in immunofluorescence experiment and the primary and secondary antibody/ antibodies used for each.

Dish labelled as	Primary antibody/antibodies used for it	Secondary antibody/ antibodies used for it
NG2	NG2	Anti-mouse and anti rabbit
No primary antibody	None	Anti-mouse and anti rabbit
ER	ER antibody	Anti-mouse and anti rabbit
NG2 and ER	NG2 and ER antibodies	Anti-mouse and anti rabbit
NG2 and Golgi complex	NG2 and Golgi antibody	Anti-mouse and anti rabbit

2.2.11 Flow cytometry

Cells were passaged, resuspended in 5ml FACS wash buffer (see appendix I) and counted. $1-2 \times 10^6$ cells/tube was used. Samples were incubated with 50µl of FACS wash and mouse serum on ice for 15 minutes. Samples were incubated with the primary antibodies (NG2 or β 1 Integrin (CD29)) or the relevant isotype controls (for NG2: PE Mouse IgG1 isotype, for β 1 Integrin: APC Mouse IgG1 isotype) for 30 minutes at 4°C. All antibodies were diluted in FACS wash. Cells were washed with 1ml FACS wash and centrifuged for 5 minutes at 717 RCF three times. Samples were mixed with 200µl FACS wash buffer and analysed using the FACS Calibur (BD).

2.2.12 Immunoprecipitation

The protein concentration of all samples used was measured before using the ABC assay determination of the protein concentrations. 100µl of sepharose A and G proteins were mixed and left on a shaker overnight before starting the procedure. Next day, the mixture of G and A sepharose proteins was washed with 15ml RIPA buffer 3xs, centrifuged for 5 minutes at 2000 rpm xg. Then, 50µls of beads was mixed with 10ml RIPA buffer and 1ml cell lysates and incubated for an hour on the shaker in the cold room. The mixture was centrifuged for 3 minutes at 1000 rpm. The supernatant was collected; 2µg/ml of rabbit anti-NG2 antibody (abcam, 86067) was added and incubated for an hour on a shaker in the cold room. Negative control (Rabbit immunoglobulin, Dako, UK) and beads only control where no antibody was added were used as controls for this experiment. After that, 50µl of beads was added to the supernatant and incubated for an hour on the shaker in the cold room. The supernatant was washed with RIPA buffer three times and centrifuged for 3 minutes at 2000 rpm xg. The pellet was transferred to 1.5ml eppendorfs; 60µl of 2X sample buffer was added and the samples were boiled for 10-15 minutes. After that, the boiled sample was centrifuged for 10 minutes at 13000 rpm xg and supernatant was collected. Samples were stored in a -80 freezer to be used later.

2.2.13 In- gel digestion and analysis of the protein bands using the Mass spectrometry

After the protein samples were run on a gel or transferred to a PVDF membrane, the gel or the membrane was washed with milli-Q water several times. The gel or the membrane was then placed in 0.25% coomassie blue stain solution (see appendix I) until the gel or the membrane was uniformly blue in colour (period is 2-4 hours).

The membrane or the gel was then destained using the destaining solution (see appendix I). The membrane or the gel was destained three times, 30 minutes each, and then the membrane or the gel was incubated in the destaining solution overnight with gentle shaking.

After the staining process was performed, the bands were cut tightly for the in-gel digestion, chopped into pieces of 1mm thickness and placed in 1ml eppendorfs. The solutions used were all freshly prepared. The bands were first incubated in 300µl of washing solution (see appendix I) at room temperature for 30 minutes. The process was repeated twice. Then the bands were incubated with 300µl of a reducing solution (see appendix I) for an hour at room temperature to reduce the protein. The bands were washed with 300µl of the washing solution three times. The protein samples were incubated with 100µl of the alkylating solution (see appendix I) at room temperature in the dark for 20 minutes. After that, protein samples were washed with the washing solution 3 times.

Protein samples were then centrifuged at 13000 rpm xg for 2 minutes and then covered with 50%ACN for 5 minutes. The samples became white at this stage. The ACN was discarded and the samples were allowed to dry at room temperature. The gel pieces were swollen by incubating with 30µl of a solution composed of 29µl ACN and 1µl of trypsin (Promega, UK) at 4°C. Initially until the gel pieces were swollen. Then samples were incubated at the 32°C for 16-24 hours. The top of the samples were sealed with the parafilm to prevent the evaporation of the solution. A blank sample was included, which contained only 29µls ACN and 1µl of trypsin without protein sample. The samples were sonicated for 10 minutes prior to spotting onto the MALDI machine.

For the analysis of the protein samples, the MALDI machine was used. The surface of the plate was cleaned with methanol or water and methanol. 0.5µl sample + 0.5µl matrix (see appendix I) were mixed and spotted into the plate. Protein samples were allowed to dry before placing in the MALDI. After that, the proteins' peaks and signals were analysed using the data explorer software.

2.3NG2/CSPG4 expression in primary human chondrosarcoma samples

2.3.1 Chondrocytes isolation from chondrosarcoma biopsy

Biopsy fragments were cut up into small pieces and incubated with 5ml trypsin for 15minutes at 37°C. Trypsin was removed and samples incubated with 20ml collagenase for 45minutes. The cell suspension was centrifuged at 707RCF for 5 minutes. The supernatant was discarded and 1ml medium added to the pellet. Cells were cultured in flasks and fed twice/week with Iscove's medium.

After the cells became confluent, protein and RNA was extracted and NG2/CSPG4 gene and protein expressions were assessed by RT-PCR and western blotting respectively. Due to the difficulty of obtaining chondrosarcoma samples, a limited number of samples were used in this study and this procedure was performed on two chondrosarcoma samples only

2.4 NG2/CSPG4 expression in different grades of OA cartilage

2.4.1RNA extraction from paraffin blocks of cartilage (OA and normal cartilage)

RNA extraction from paraffin blocks of the cartilage was performed following the Qiagen procedure. The excess paraffin was trimmed off the sample block using a scalpel. Then cartilage sections were cut (5–10µm) thick. Sections were placed in a 2ml microcentrifuge

tube. The RNA extraction was started by the addition of 1ml xylene to the sample, vortexed vigorously for 10 seconds and centrifuged at 13000 rpm xg for 2 minutes at 20–25°C. The supernatant was removed by pipetting using large tip and placing the small tip on the top of it. After that, 1ml of 100% ethanol was added to the pellet, vortexed and centrifuged at full speed for 2 minutes at 20–25°C. The supernatant was removed by pipetting, the lid of the tube was kept open and incubated at room temperature (15–25°C) for 10 minutes or until all residual ethanol has evaporated. A the hot plate was set up to 55°C. Then the pellet was resuspended in 240µl Buffer PKD mixed with 10µl proteinase K and vortexed. Samples were incubated at 55°C for 15 minutes, then at 80°C for 15 minutes. 500µl Buffer RBC was added to samples to adjust binding conditions. The lysate was mixed and transferred it to a gDNA Mini Elute spin column, which was placed in a 2ml collection tube. The sample was centrifuged for 30 seconds at 13000 rpm xg. The step was repeated until no liquid remained on the column membrane. After that, the column was discarded and the flow-through was saved.

After the above, 1200µl of 100% ethanol was added to the flow-through, and mixed well by pipetting. A sample of 700µl was transferred, including any precipitate that may have formed, to an RNeasy Mini Elute spin column placed in a 2ml collection tube. The sample was centrifuged for 15 seconds at $\geq 8000 \times g$ ($\geq 10,000$ rpm) and the flow through was discarded. The step was repeated until the entire sample has passed through the RNeasy Minutes Elute spin column. 500µl of Buffer RPE was added to the RNeasy Mini Elute spin column and centrifuged for 15 seconds at 13000 rpm to wash the spin column membrane. The flow-through was discarded and 500µl Buffer RPE was added again to the RNeasy Mini Elute spin column. The RNeasy Mini Elute spin column was placed in a new 2ml collection tube and the old collection tube was discarded with the flow-through. The lid of the spin

column was opened, and centrifuged at 13000 rpm xg for 5 minutes. The collection tube was discarded with the flow-through and the RNeasy Mini Elute spin column was placed in a new 1.5ml collection tube. 15µl RNase-free water was added directly to the spin column membrane and centrifuged for 1 minute at 13000 rpm xg to elute the RNA. The supernatant was collected into a new tube. RNA amount was assessed using the Nano Drop technique. Next, RNA was stored at -20 until used. After that, RNA extracts were used to investigate NG2/CSPG4 expression by qPCR as described earlier in this chapter.

2.5 NG2/CSPG4 gene knockdown in JJ012 human chondrosarcoma cell line

2.5.1 MTT assay to assess the cytotoxic effect of hexadimethrine bromide in JJ012 cells

The recommended dose range of hexadimethrine bromide is (1, 2, 4 and 8µg/ml). Cells were grown in 96 well plates at 1×10^4 /well cell density. Twenty four hours later, cells were treated with different doses of hexadimethrine bromide (2mg/ml) (1, 2, 4 and 8µg/ml) for 24 hours and assessed by MTT.

2.5.2 Establish the puromycin titration (killing curve)

Puromycin is used as a selective indicator for the knockdown colonies. This should be performed whenever working with a new cell line. JJ012 cells were grown at a 2.5×10^4 /well /100µl into the 96 well plates and grown for 24 hours in the incubator at 37°C. Next day, puromycin (10 mg/ml stock solution, Sigma, UK) was added to wells at 0.5, 1, 2 and 4µg/ml. 20µl of the alamar blue dye (AbD Setrotec, UK) was added to wells and plate at 750 and 600 absorbance. The readings were taken at 4, 24 and 48 hours respectively. If the concentration

for the desired cell type is unknown, a titration experiment must be performed. Typically, 2-10 µg/ml are sufficient to kill most untransduced mammalian cell types.

2.5.3NG2/CSPG4 knockdown using the Lentiviral Transduction

The mission TRC shRNA libraries are lentiviral based shRNA vector collections for use in gene knockdown studies. This provides a system for long-term silencing and phenotypic observation. MISSION TRC shRNA lentiviral particles target set (2-15 ml per shRNA construct), negative control (shRNA non-target control) (SHC002V) together with the positive control (shRNA GFP control) were used in this experiment. The non-target control contains 4 base pair mismatches within the short hairpin sequence to any human genes. It is useful as a negative control in the experiments with MISSION shRNA target sets.

Cells should be no more than 70-80% confluent before transduction. On Day 1, JJ012 cells were seeded at 0.625×10^4 in 96 well plates and grown for 24 hours in a humidified incubator at 37°C supplemented with 5% CO₂. On **Day 2**, the media was removed from wells and replaced with fresh media. At the same time, lentiviral particles were added to wells in a 10 folds dilution. Thus, 20µls of viral particle was added to the well 1, mixed thoroughly, 10µls taken from well 1 and added to well 2. This step was repeated until the last well in the plate (well 12). Five different constructs against NG2/CSPG4 were used (A, B, C, D and E) together with the controls (GFP and the non target). The procedure was carried out in duplicate. On **Day 3**, the media was removed from wells and replaced with fresh media to allow cells to recover. On **Day 4**, the media was removed from wells and replaced with media containing puromycin. From **Day 5 on**, the media was removed from wells and replaced with fresh media containing puromycin every 2-3 days. At the same time, cells were checked whether they became confluent and resistant colonies identified. To identify resistant

colonies, cells were examined under the microscope from well 1 to 12, to investigate where cells were still alive (spreading) or dead (rounded) and marked wells where no cells which were found alive.

Sometimes, one colony was identified/well; while a range of 2-4 colonies were identified in other wells. Due to the random integration of the lentivirus into the host genome, varying levels of target gene knockdown may be seen with different puromycin resistant colonies. Testing a number of colonies will allow the optimal degree of knockdown to be determined. Once cells became confluent, wells with the highest amount of viral particles (where 20µls of viral particles added, known as pooled cells) are passaged according to the protocol, transferred into 25cm² flasks and then expanded into 75 cm² flasks. Finally, cells were frozen down (see protocol above) and the level of gene knockdown was investigated by qPCR. When cells grown in other wells became confluent, they were passaged and expanded depending on the number of colonies identified. When one colony was found, cells were passaged, grown in 58mm dish, expanded into 100mm Petri dishes (Nunc, UK) and then frozen down. While if more than one colony was identified, cells were treated in the same way as noted above, expanded into 100mm Petri dishes (Nunc, UK) and then cloning rings were used for the selection of the colonies.

The selection of colonies can be done using cloning rings. For the selection of the colony, cells were expanded into 100mm Petri dishes (Nunc, UK) and colonies were identified and their areas were marked with the pen on the bottom surface of the dish. Then, media were removed, cloning rings were placed in the dish where the marked area, trypsin/EDTA was added and incubated for 3 minutes. Then, cells were transferred and mixed with fresh media. Cells were expanded for 2-3 passages from 25 to 75 cm³ and a range of 5-10 colonies were

selected/construct. RNA was extracted from the cells of selected colonies, assessed for the gene knockdown by qPCR and the rest of cells were frozen down and stored at -20°C for one day before being moved to the liquid nitrogen for long term storage.

Table 2.4: The table below shows the sequence of the five different constructs used for NG2/CSPG4 knockdown in JJ012 chondrosarcoma cell line.

Construct name	Sequence
A	CCGGCAATGCATCAGCCGTAGTGAACCTCGAGTTCACTACGGCTGATGCATTGTTTTTG
B	CCGGCATCACGGTGAGGGATGTAACTCGAGTTTACATCCCTACCGTGATGTTTTTG
C	CCGGCTTTGCCACTGAGCCTTACAACCTCGAGTTGTAAGGCTCAGTGGCAAAGTTTTTG
D	CCGGCAACATGTTTCAGCGTCATCATCTCGAGATGATGACGCTGAACATGTTGTTTTTG
E	CCGGGACTTCATCTATGTGGACATACTCGAGTATGTCCACATAGATGAAGTCTTTTTTG

2.6 Study the functional roles of NG2/CSPG4 in human chondrocytes

2.6.1 Treatment of JJ012 cells with chemotherapeutic drugs

To optimise the seeding density and the time point to treat JJ012 cells with chemotherapeutic drugs, cells were seeded at various densities (2.5×10^4 , 1.25 and 0.625×10^4) in 96 well plates in triplicate (100µl/well). Twenty four hours later, medium was changed and cells were grown for 24, 48 and 72 hours. Cell growth was assessed by MTT assay.

For dose response curves, cells were seeded at 1.25×10^4 and treated with different doses of chemotherapeutic drugs (see table 2.4 below) for 48 hours. The effect of the drugs was assessed by MTT assay.

Table 2.5: The table below demonstrates the use of different doses of chemotherapeutic drugs to treat JJ012 (NG2+ve) and B3 (NG2-ve) cells

Drug	Vehicle control	Treatment				
Doxorubicin	DMSO	0.03 μ M	0.01 μ M	0.1 μ M	0.3 μ M	1 μ M
Ifosfamide	DMSO	0.3mM	1m M	3 mM	10 mM	30 mM
Docetaxel	Ethanol	1nM	3nM	10nM	30nM	100nM
Cisplatin	H ₂ O	1 μ M	3 μ M	10 μ M	30 μ M	100 μ M

2.6.2 MTT assay

2-3 hours before the end of the treatment, 20 μ l of MTT was added. Medium was removed and 100 μ l of DMSO was added. The plate was read at 550nm absorbance using the Biotech spectrophotometer.

2.6.3 Flow cytometry for Annexin V staining

Annexin V staining Flow cytometry was used to assess the percentages of apoptosis in JJ012 (NG2+ve) and B3 (NG2-ve) cells. Cells were cultured in 75 cm³ flasks and treated as required. All the tubes required were prepared (falcon and flow tubes) and labelled. At the of the treatment time point, the supernatant was removed and put in falcon tubes because this contained cells which were dead and floating and 1-2ml Trypsin/EDTA was added to the flask and incubated for 3-4 minutes in the incubator. Then, cells were passaged according to the protocol described early in this chapter. PBS was then added to the flask and the supernatant was collected (50ml/tube). Cells were resuspended in 5ml Hanks balanced salt buffer (containing 500um 1M CaCl₂) and a cell count was performed. 1-2x10⁶ cell density/1ml/ tube was used. Cells were centrifuged for 5 minutes at 2000 rpm xg. Hank's Buffer was discarded by gently flicking the tubes where cell pellets should not be disturbed. The tubes were gently tapped on a tissue to remove any remnants buffer. The annexin V

(1mg/ml) was added to the tubes (400µl Hanks buffer+ 1µl annexin V/tube). Cells were incubated on ice for 10-15minutes. For propidium iodide (PI) use, 1mg/ml was added immediately before analysing samples (400µls Hanks buffer+1µl PI/tube). Cells were analysed using the FACscan machine (BD, UK). Four samples were prepared for each treatment; unstained, Annexin V only, Annexin V and PI and PI only. Sometimes, two flasks/treatment were prepared to ensure that sufficient cell numbers were collected.

2.6.4 Cell adhesion assay

Methylene blue adhesion assay was used to measure the difference in cell adhesion between JJ012 (NG2+ve) and B3 (NG2-ve) cells. To set up the standard curve for methylene blue adhesion assay, JJ012(NG2+ve) and B3(NG2-ve) cells were passaged and seeded at different densities (0.625 , 1.25 , 2.5 and 5×10^4) in triplicate in 96 well plates. Cells were grown for 1 hour at 37°C in the incubator. Cells were washed with PBS twice to remove non-adherent cells gently without touching the bottom of the plate. Cells were fixed with $100\mu\text{l}$ / well of 4% formalin in BPS and incubated for 1 hour. After that, cells were centrifuged on the plate at 1000 rpm $\times g$ for 5 minutes and $100\mu\text{l}$ /well of pre-filtered 1% methylene –blue was added and incubated for 30 minutes. After that, the plate was washed 3 times with PBS, BPS was removed by suction and cells were centrifuged for 5 minutes at 2000 rpm $\times g$ to remove the excess blue dye. $100\mu\text{ls}$ of the 0.1N HCL in distilled water was added to wells, mixed thoroughly and incubated for 5 minutes, ensuring that all the particles of the dye are evenly distributed. The plate was read at 650nm . The number of adherent cells is proportional to the amount of dye bound which is proportional to the absorbance at 650nm .

To assess the cell adhesion to different ECM molecules, the plates were coated at 10µg/ml of ECM proteins (Collagen I, II, VI and FN) together with the negative control (BSA) in 50µls PBS, overnight at 4 degree. Next day, the blocking solution (BSA, 10mg/ml) (see appendix I) was added for 1 hour at 37°C. Uncoated wells were used in this experiment. Cells were passaged and seeded at 2.0×10^4 / 100µl in triplicate in 96 well plates. Cells were grown for an hour at 37 °C in the incubator. When integrin blocking antibodies, EDTA (5 and 10mM) or EGTA (5 and 10mM) were used, cells were incubated for 30 minutes at 37°C in the incubator prior to seeding. Then, the procedure was carried out as described above.

2.6.5 Cell spreading assay

The plates were coated with 10µg/ml of ECM proteins (Collagen, I, II, III, VI, V and FN) in 50µls PBS at 4°C overnight. Next day, the blocking solution was added to wells (BSA 10mg/ml) and incubated for an hour at 37°C. Wells which were uncoated and incubated with BSA were used. JJ012 (NG2+ve), B3 (NG2-ve) and non-target cells were passaged as described before and seeded in triplicate in 96 well plates. 2×10^5 / ml (2×10^4 /100µl /well) was the seeding density used. Cells were allowed to grow for an hour in the incubator at 37 °C. After that, media was removed from wells and cells were washed twice with PBS to remove the non-adherent cell. The washing step was performed gently, trying not to touch the bottom of the plate. Then, 100µls of 4% formalin in PBS was added to wells and incubated for an hour at RT. Cell were then imaged using a bright field microscope. Images were taken from at least three independent areas/well and the spreading cells were counted. There are different criteria stated on how to differentiate between spreading and non-spreading cells. The cell spreading assay was performed following the criteria of cell spreading used by Martin J Humphries. Cells should be regarded as spreading cells when the cell body is phase dark and the cytoplasm should be visible around the entire circumference of the nucleus.

2.6.6 Wound healing assay to assess the cell migration

The wound healing assay was used to assess the cell migration in JJ012 (NG2+ve) and B3 (NG2-ve). Cells were seeded in 12 well plates at 7.5×10^4 / well and grown in monolayer at 37°C in a humidified incubator with 5% CO₂ until confluency. A few hours later, cells were removed from the incubator, media was removed and wells were wounded by micropipette at the middle of the wells. Then cells were washed with DMEM media and fresh media specific for JJ012 added. Cells were imaged using the bright field imaging microscope (Olympus, UK). Images of the wounded area were taken immediately and after 10 hours of the scratch. The percentage of area closed was assessed using T scratch software for both cell types. For statistical analysis, unpaired T test was used to investigate whether there is any significant difference in cell migration between the two cell types, with p value <0.05 is considered to be statistically significant.

2.6.7 CyQUANT® Cell Proliferation Assay

Cell proliferation was carried out using CyQUANT® Cell Proliferation Assay kit (C35007, invitrogen, UK). JJ012 (NG2+ve), B3 (NG2-ve) and non-target cells were passaged as described before and seeded in triplicate in 96 well plates. Five thousand cells/ well was the seeding density used. Cells were allowed to grow for 24 and 48 hours in the incubator at 37°C. At the end of the time point, the growth medium was removed from cells by gentle aspiration using a manual multichannel pipettor without disturbing the cells. Prepare 1X dye binding solution (provided in the kit) was prepared by adding 22µl of CyQUANT® NF dye reagent (Component A) to 11ml of 1X HBSS buffer. Then, 100µl of 1X dye binding solution (already prepared) was added into each well using a manual multichannel pipettor. The plate was covered with tin foil and incubated at 37°C for 15 minutes. At the end of the incubation, the fluorescence intensity of each sample was measured using the fluorescence microplate

reader with excitation at 485nm and emission detection at 530nm. The measurements were carried out at RT.

2.6.8 Inverted cell invasion assay

An aliquot of Matrigel (growth factor reduced Matrigel) (BD Bioscience, UK) was thawed slowly on ice and diluted as 1:1 in ice cold PBS. 100µl of the matrigel was pipetted carefully into each transwell (inserted into a well of a 24 well tissue culture plate) and incubated at least 30 minutes at 37°C to allow it to set. During that, cell suspensions were prepared at 1×10^5 cells per ml in their normal growth medium including serum. When Matrigel was set, transwells were inverted and 100µl of the cell suspension was pipetted into the underside of the filter (which is now uppermost). The transwells were covered carefully with the base of the 24 well tissue culture plate such that it contacted the droplet of cell suspension and incubated for 4 hours to allow the cells to attach. After that, the plate was turned right-side-up and each transwell was dipped sequentially into 3x 1ml serum free medium to wash. This was most easily done with wash 1 being in the original plate and washes 2 and 3 being in a fresh plate. The transwell was left in wash 3 as this served the well in which the assay was to be carried out. Then, 100µls of either: serum containing media or serum containing media + EGF (Epidermal Growth Factor) was pipetted gently into the transwell on top of the matrigel. The lid was replaced and cells were incubated 37°C for 5 days. On Day 5, cells were stained with Calcein 4µM (Invitrogen, UK) (see appendix I). 0.5ml of Calcein staining in PBS was added to wells of 24 well plates, a transwell was added to each well and 0.5ml of the solution into the top of each transwell. The cells were incubated for an hour at 37°C and wells were imaged using the confocal microscope.

Stained nuclei or whole cells were visualised by confocal microscopy using a 20X objective. Optical sections (Z-sections) were scanned at 15µm intervals (Z-steps) moving up from the underside of the filter into the Matrigel, producing a series of images. To quantify these, “Image J” software can be used. Essentially, this programme gives each on-screen pixel an intensity value from 0 through to 255. The background pixel value is then operator-defined as the point (pixel value) on the 0-255 scale at which only cells from that individual section are visible with no bleed-through from bordering sections. Only pixels with an intensity value greater than background are then counted by “Image J”. For quantification purposes, only cells in the 45µm section or above were considered invasive. Either a compound image of these sections can be created in “Image J” and quantified or each image can be quantified separately and the sum of the values obtained can be used. This value was then normalised to the value obtained from the corresponding 0µm section as a “loading” control.

2.7 Statistical analysis

The average, standard deviation and standard error of the mean (SEM) were determined in each experiment. In each experiment, the samples were tested whether they are normally distributed or not. Student unpaired t-test was used to find out the statistically significant difference between two independent groups. For three independent groups, One-way ANOVA was used. The difference was considered statistically significant when $p < 0.05$.

Table 2.6: The table below shows the details of all primary antibodies used in this study and the experiments used for.

Antibody	Supplier	Catalogue number	Clone	Experiment used (concentration)
NG2	Santa Cruz	sc-53389	LHM 2	<ul style="list-style-type: none"> • Western blotting(1µg/ml) • Immunofluorescence (4µg/ml) • IHC (20µg/ml)
NG2	Santa Cruz	sc-80003	9.2.27	<ul style="list-style-type: none"> • Western blotting(1µg/ml)
NG2	Santa Cruz	sc-53389PE	LHM 2	<ul style="list-style-type: none"> • Flow cytometry(20µg/ml)
NG2	BD Bioscience	554275	9.2.27	<ul style="list-style-type: none"> • Western blotting(1µg/ml) • Flow cytometry(20µg/ml)
NG2	Abcam	ab86067	Amino acids 575-625 of Human NG2	<ul style="list-style-type: none"> • Immunoprecipitation (20µg/ml)
Anti-Calnexin - ER membrane marker antibody	Abcam	ab10286	N-terminal ER luminal domain of canine calnexin	<ul style="list-style-type: none"> • Immunofluorescence (1/50 dilution)
Anti-Giantin	Abcam	ab24586	N terminal amino acids 1-469 of Human Giantin.	<ul style="list-style-type: none"> • Immunofluorescence (1/50 dilution)
β1 integrin	BD Bioscience	559883	MAR4	<ul style="list-style-type: none"> • Flow cytometry(20µg/ml)
Tubulin	Abcam	ab56676	Amino acids 1-452 of Human Tubulin	<ul style="list-style-type: none"> • Western blotting (0.1µg/ml)
β1 integrin blocking	Millipore	MAB1987Z	P4C10	<ul style="list-style-type: none"> • Cell adhesion assay (1mg/ml).
αVβ3 integrin blocking	Millipore	MAB1976Z	LM609	<ul style="list-style-type: none"> • Cell adhesion assay (1mg/ml).

Table 2.7: The table below shows the details of all the secondary antibodies used in this study and the experiments used for.

Antibody	Supplier	Catalogue number	Experiment used (concentration)
Rabbit anti mouse HRP	Dako	P0260	<ul style="list-style-type: none"> • Western blotting (13µg/ml)
PE Mouse IgG1 isotype	BD bioscience	550617	<ul style="list-style-type: none"> • Flow cytometry(20µg/ml)
APC Mouse IgG1 isotype	BD bioscience	554681	<ul style="list-style-type: none"> • Flow cytometry(20µg/ml)
AlexaFluor 488 goat anti-mouse	Invitrogen	A11070	<ul style="list-style-type: none"> • Immunofluorescence (10µg/ml)
AlexaFluor 647 goat anti—rabbit IgG	Invitrogen	A21244	<ul style="list-style-type: none"> • Immunofluorescence (10µg/ml)

Table 2.8: The table below shows the details of all primers used in this study.

Primer	Sequence 3'-5'	Product Size	Cycle conditions	Primer source
GAPDH	TCTAGACGGCAGGTCAGG TCC ACC CCACCCATGGCAAAT TCCATG GCA	600 bp	93°C 3m 93°C 15s 58°C 30s 72°C 90s X40 72°C 10m	Invitrogen, UK
18s	Custom made primer	99bp	95°C 2m 95°C 1m 60°C 1m x40 72°C 1m 72°C 10m	Primer design, UK
RPLA13	Custom made primer	223bp	95°C 2m 95°C 1m 60°C 1m x40 72°C 1m 72°C 10m	Primer design, UK
CO1	CTA TAG TGG AGG CCG GAG CAG GAA GAG GGG GCT GTT GGT ATT GGG TTA TG	201bp	95°C 2m 95°C 1m 60°C 1m x40 72°C 1m 72°C 10m	Invitrogen, UK

NG2	GGCTGTCAA AAC CAG GGT AA CTT CCTTCTCCTTGCCCTCT	113bp	95°C 2m 95°C 1m 60°C 1m x40 72°C 1m 72°C 10m	Invitrogen, UK
Aggrecan	TGAGGAGGGCTGGAACAAGTACC GGAGGTGGTAA TGCAGGGAA CA	346bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
Collagen II	AGGAGGCTGGCAGCTGTGTGC CACTGGCAGTGGCGAGGTCAG	207bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
SOX-9	GCGGAGGAAGTCGGTGAAGA TTGGAGATGACGTCGCTGCT	444bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK

RUNX-2	CAGACCAGCAGCACTCCATA CAGCGTCAACACCATCATTC	177bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
Decorin	AGGCGTCCTGTGGCAATT TCAGATGAGGCGTGTTGG	198bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
MMP3	AGATGATATAAATGGCATTTCAG CTCCAACGTGAAGATCCAG	614bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
MMP13	GGCTCCGAGAAATGCAGTCCTTCTT ATCAAATGGGTTAGAAGTCGCC ATGC	347bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 72°C 10m	Invitrogen, UK

ADAMTS-4	ACCACCCTGACCACTTTGACACA GCCATTGACCTGGCCAGA		95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
ADMATS-5	TGACCATGAGGAGCACTACG TGGGAGAGGCCAAGTAAATG	198bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
IL-1B	AAACAGATGAAGTGCTCCTTCCAGG TGGAGAACACCACTTGTTGCTCCA	390bp	95°C 2m 95°C 1m 55°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
IL2RC	CTCCTTGCCTAGTGTGGATGG CACTGTAGTCTGGCTGCAGAC	255bp	95°C 2m 95°C 1m 62°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK

IL4R	CTTGTTACCTTTGGACTGG CTTGAGCTCTGAGCATTGCC	454BP	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
IL10R	CAGTCTGAGAACAGCTGCACC GATGTCAAACCTCACTCATGGC	416bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
IL13R alpha 1	TGCAATGGGAGAATCCACAG AGGAAGAACACCAGGGACCA	179bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
IL13R alpha 2	TGGGACCTATTCCAGCAAGG AGGTCTTCACCTTCCCAGCA	223bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
HSP70	GAGCTGTGCTCCGACCTGTT CGGGGTTGATGCTCTTGTTTC	187bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK

GRP78	ACGGGCAAAGATGTCAGGAA AATTCGAGTCGAGCCACCAA	285bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
PDI	TTGGCCTCACCAAGGACACT GGCTGTTGAACTCCGTGACC	153bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
Bag-1	ATGAATCGGAGCCAGGAGGT GGCCAGGTCTTGGACAACCTG	177bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
COL6 alpha	GTCTTCTCGGTGGCCATCAC CTTGCCTCGTTCTCCCTCAA	264bp	95°C 2m 95°C 1m 58°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
PDGFA	GCTGCAACACGAGCAGTGTC CCTGACTCCCTAGGCCTTCC	205bp	95°C 2m 95°C 1m 58°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK

PDGFB	GCCTCATAGACCGCACCAAC ACTTGCATGCCAGGTGGTCT	213bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
TIMP	GACACCAGAAGTCAACCAGACC GACTGCCAGGTGCACAGCCC	458BP	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK
FGF2	GGAGAAGAGCGACCCTCACA GCCCAGGTCCTGTTTTGGAT	269bp	95°C 2m 95°C 1m 60°C 1m X35 72°C 1m 72°C 10m	Invitrogen, UK

CHAPTER THREE

Results

3.0 NG2/CSPG4 expression in human chondrocytes

3.1 NG2/CSPG4 expression *in vivo*

3.1.1 NG2/CSPG4 expression *in vivo* using immunohistochemistry

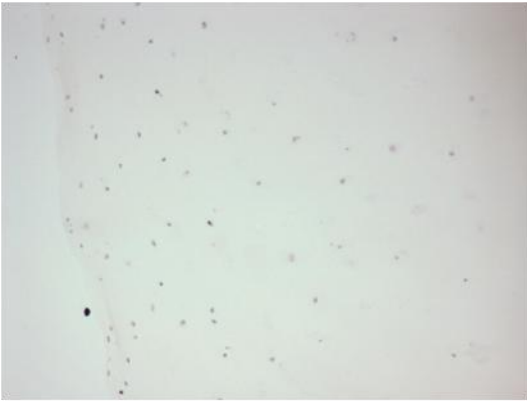
It was necessary first to determine whether NG2/CSPG4 is expressed in diseased cartilage tissues *in vivo*. Immunohistochemistry was performed on frozen sections from 7 human chondrosarcoma samples of different grades (details shown in the table 3.1 below). Frozen sections from OA cartilage was used as positive controls. Negative controls were prepared by omitting the primary antibody incubation step.

Results showed that there was a positive immunoreactivity for NG2/CSPG4 in all human chondrosarcoma samples used. Figure 3.1 of the results shows a representative image for immunohistochemistry results on human chondrosarcoma samples.

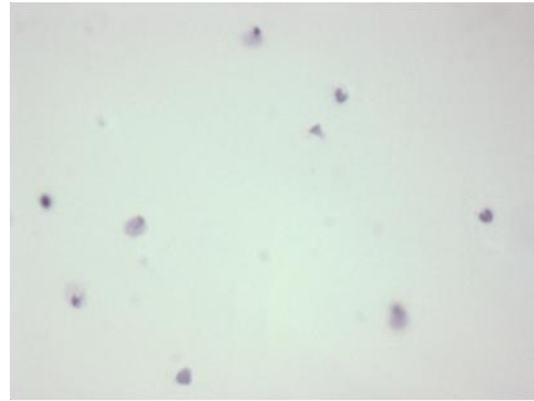
Table 3.1: Details of human chondrosarcoma samples used in immunohistochemistry and qPCR experiments to investigate NG2/CSPG4 expression *in vivo*.

Sample number	Source	Age	Sex	Gross pathological features	260/280 ratio obtained by Nanodrop spectrophotometer after RNA extraction
14292	Western General Hospital, Edinburgh	32 years	Male	Grade II chondrosarcoma	Insufficient material to extract RNA. Used in immunohistochemistry.
14307	Royal Infirmary, Edinburgh	62 years	Female	Grade II chondrosarcoma	2.1
S00018088	Orthopaedic Department, Oxford	43 years	Female	low-grade chondrosarcoma arising within a pre-existing enchondroma	1.56
S00018180	Orthopaedic Department, Oxford	46 years	Female	Grade II chondrosarcoma showing areas of myxoid change and focal areas of necrosis	1.62
S00018387	Orthopaedic Department, Oxford	63 years	Female	Low-grade chondrosarcoma	1.50
S0007752	Orthopaedic Department, Oxford	79 years	Male	Grade II chondrosarcoma	1.43
S00043887	Orthopaedic Department, Oxford	53 years	Male	Grade 0.5 chondrosarcoma	1.54

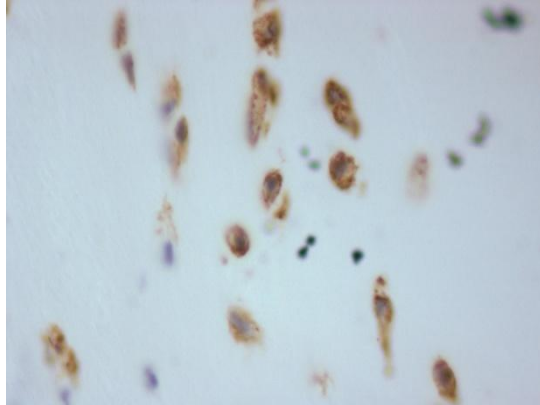
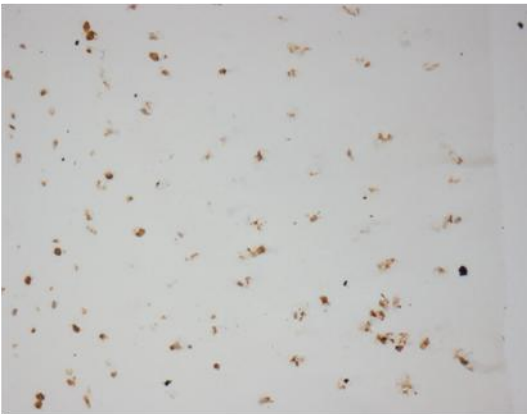
(a)



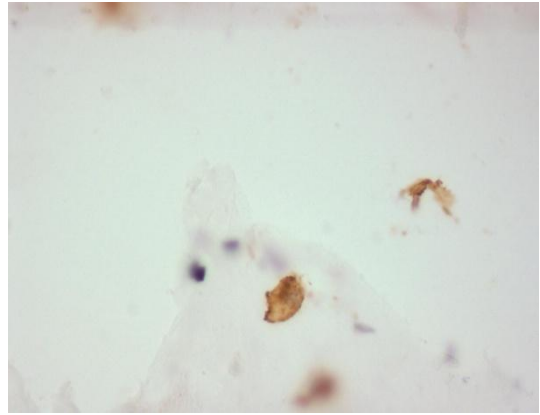
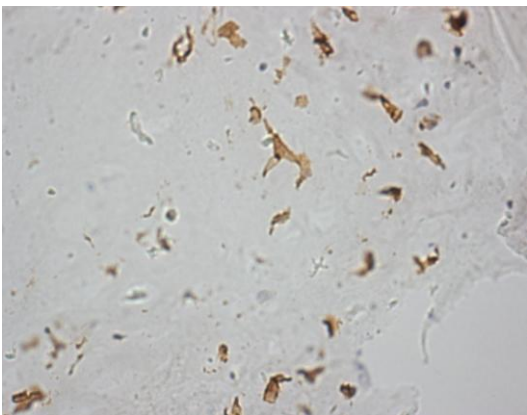
(b)



Negative control



OA Cartilage



Chondrosarcoma

Figure3.1: Representative images (n=7) for immunohistochemistry results for NG2/CSPG4 expression in human chondrosarcoma (frozen sections). OA cartilage (sample number 14301) was used as positive control. Negative control was obtained by omission of the primary antibody. The images in column (a) represent images taken at 20 xs and images in column (b) represent images were taken at 40x.

3.1.2 NG2/CSPG4 expression *in vivo* using qPCR

RNA extraction was carried out on frozen sections of 6 out of 7 human chondrosarcoma samples of different grades (same samples used for immunohistochemistry experiments). RT products were prepared from these samples and then used to investigate NG2/CSPG4 gene expression by qPCR. There was insufficient frozen material from sample number (14292) to perform RNA extraction and subsequent RT and qPCR reactions.

NG2/CSPG4 expression of each individual sample was normalised to the housekeeping gene (HMT-CO1). Results showed that NG2/CSPG4 gene expression was detectable in all samples tested as shown in figure 3.2. There was no difference in NG2/CSPG4 gene expression level between different grades of human chondrosarcoma samples used; however, further human chondrosarcoma samples from different grades will be required to support these data and accurately assess whether there is a difference in the level of mRNA expression between different grades of chondrosarcoma.

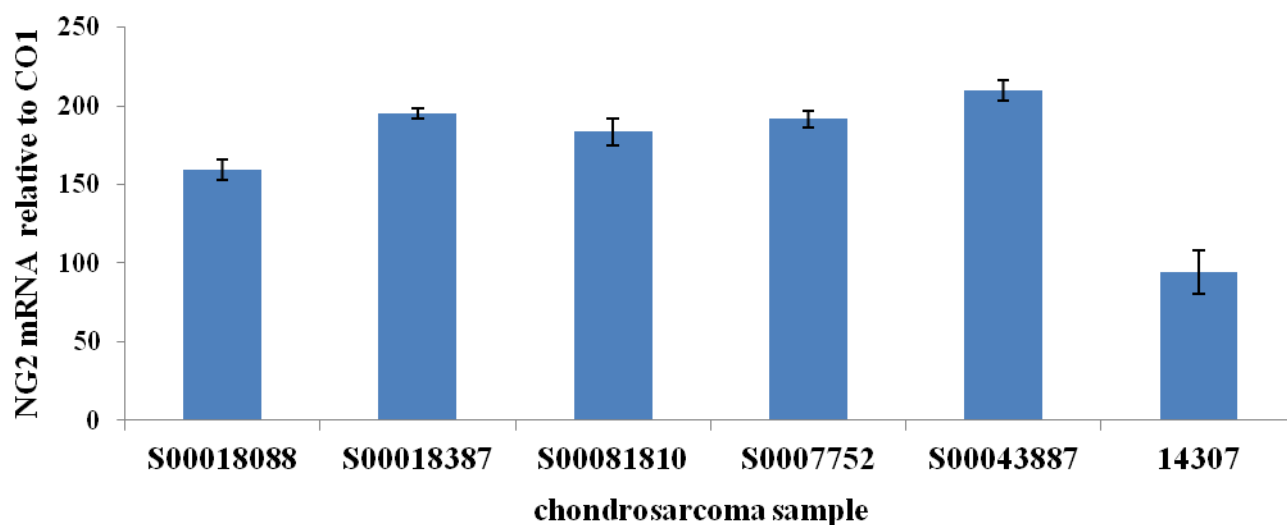


Figure 3.2: qPCR results for NG2/CSPG4 expression in 6 out of 7 human chondrosarcoma (frozen sections, different grades). The grades of the chondrosarcoma samples are as follows: 14307(II), s0001808 (low, arising in a pre-existing area of enchondroma), S00018387 (low, with an area of myxoid changes and focal necrosis), S00081810 (low), S0007752 (II) and S00043887 (0.5). The level of NG2/CSPG4 expression was normalised to CO1. There were insufficient materials to do RNA extraction for sample 14292. Error bars represent SEM of the triplicate wells/sample.

3.2 NG2/CSPG4 expression *in vitro*

3.2.1 Gene expression

RNA extracts from different cells used in this study (JJ012, primary human articular chondrocytes and C₂₀A₄ chondrocytes cell line) were used to investigate NG2/CSPG4 gene expression by qPCR and RT-PCR reactions.

A. Assessment of RNA integrity and genomic DNA contamination

RT-PCR reaction involves analysis of small regions of RNA, and therefore is tolerant to partially degraded RNA. To check for RNA integrity, RNA extracts were run at 1.5% agarose gel. Good quality RNA should show two intense bands at 150bp and 250bp. Image (a) in figure (3.3) shows one example for this test performed on OA chondrocytes showing such bands.

To check for genomic DNA contamination, cDNA was mixed with loading dye and run on 1.5% agarose gel. Image (b) in figure (3.3) is a representative image of a genomic DNA contamination test on 8 RNA extracts from JJ012 cells. It illustrated that there were multiple bands in RNA extracts tested. This means that RNA extracts were contaminated with genomic DNA and non-specific binding appeared. According to this result, an Optional On-Column DNAase Digestion was used in all subsequent RNA extractions.

B.NG2/CSPG4 expression by qPCR and RT-PCR reactions

NG2/CSPG4 mRNA expression was examined by RT-PCR in JJ012, OA chondrocytes, chondrocyte cell line and normal chondrocytes. NG2/CSPG4 expression was normalised to the housekeeping gene (GAPDH). qPCR Results showed that NG2/CSPG4 gene expression was detectable in all cells tested. Ten out of ten human OA samples, 6 out of 6 JJ012 and chondrocyte cell line RNA extracts (figure 3.4 and 3.5). However, there was no statistical difference in the level of NG2/CSPG4 gene expression between JJ012, chondrocyte cell line and OA chondrocytes.

Using the same samples used above in qPCR reaction, NG2/CSPG4 gene expression was detected in all samples tested.

Table 3.2:

a) Details of OA samples used to investigate NG2/CSPG4 expression by RT- PCR and qPCR.

Sample number	Age	Sex	OA grade	Number of days in culture
14299	61 years	Female	Not known	15 days
14300	64 years	Female	Not known	14 days
14301	50 years	Female	Not known	11 days
14302	77 years	Female	III	13 days
14303	71years	Male	III	14 days
14308	62years	Female	I-II	11 days
14312	73 years	female	Not known	16 days
14316	73 years	Male	I-II	13 days
14318	65 years	Male	I-II	12 days
14319	85 years	Female	Not known	10 days

b) Details of normal human cartilage samples used to investigate NG2/CSPG4 expression by RT-PCR.

Sample number	Age	sex	OA grade	Number of days in culture
14282	29 years	Male	0	11 days
14286	39 years	Male	0	14 days

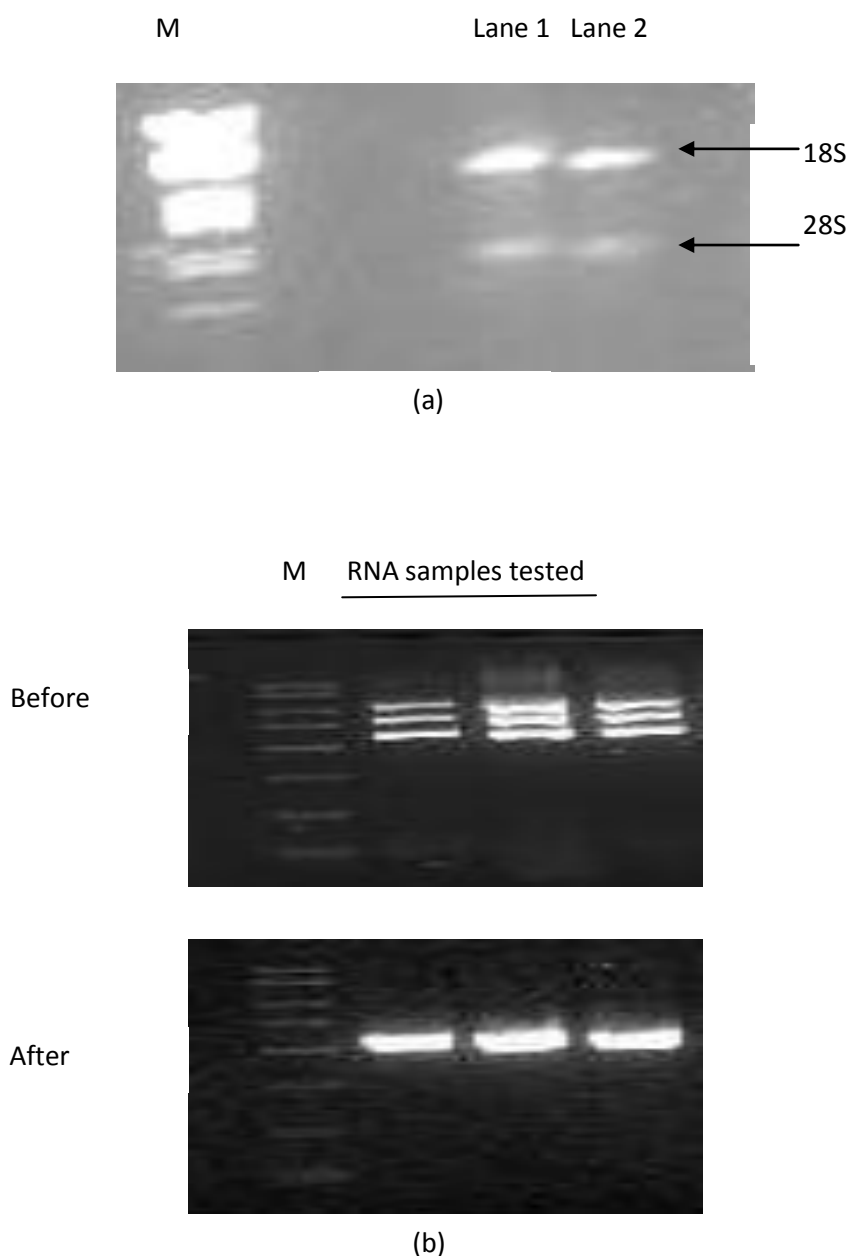


Figure 3.3: (a) Representative image for RNA integrity test. (b) Genomic DNA contamination test. M represents PCR marker. In image (a), Lane 1=OA sample (14300). Lane 2=OA sample (14301). The two bands shown in the image are: 28S and 18S human ribosomal RNA bands (28S is at twice the intensity as 18S), which indicates the integrity of the RNA. Image (b) represents 3 RNA samples tested before and after genomic DNA removal in JJ012 cell line at different passage numbers (15, 16 and 20 respectively). Before using an Optional On-Column DNAase Digestion in RNA extraction, there were multiple bands in RNA extracts tested, which means RNA extracts were contaminated with genomic DNA and non-specific binding appeared. When an Optional On-Column DNAase Digestion was used in RNA extractions, only one band appeared.

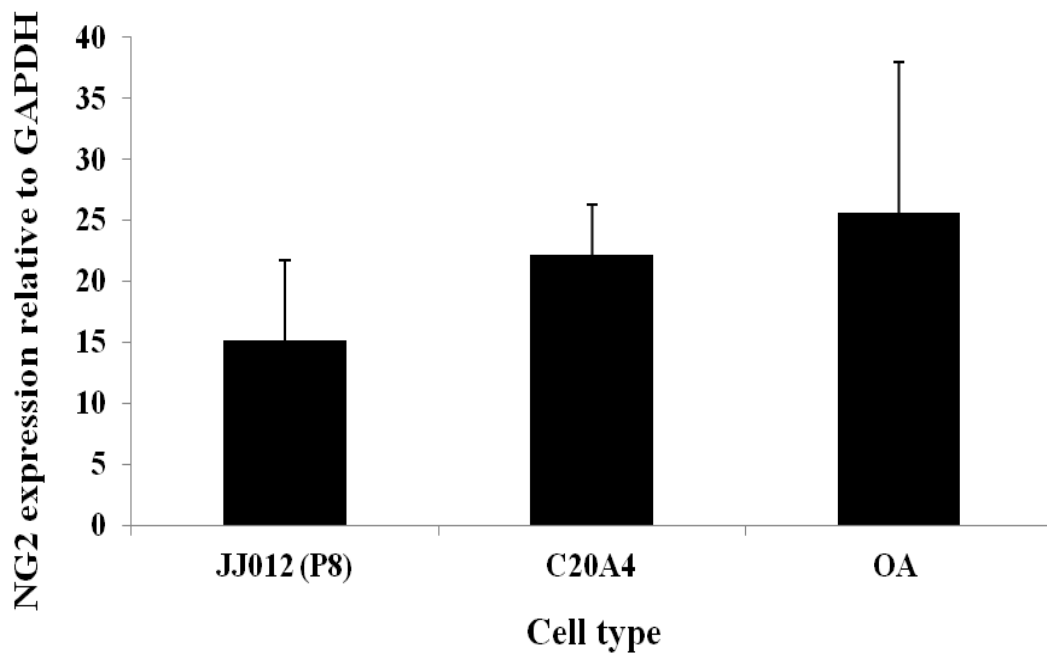


Figure 3.4: qPCR results for NG2/CSPG4 expression in primary human chondrocytes (n=6 for JJ012 and C₂₀A₄ and n=10 OA samples). Values shown represent the mean±/-SEM. Results were normalised to GAPDH housekeeping gene. OA samples were expanded *in vitro* and their details are shown in table 3.2. JJ012 and C₂₀A₄ cell lines were used at different passages. There was no statistical difference in the level of NG2/CSPG4 gene expression between JJ012, chondrocyte cell line and OA chondrocytes.

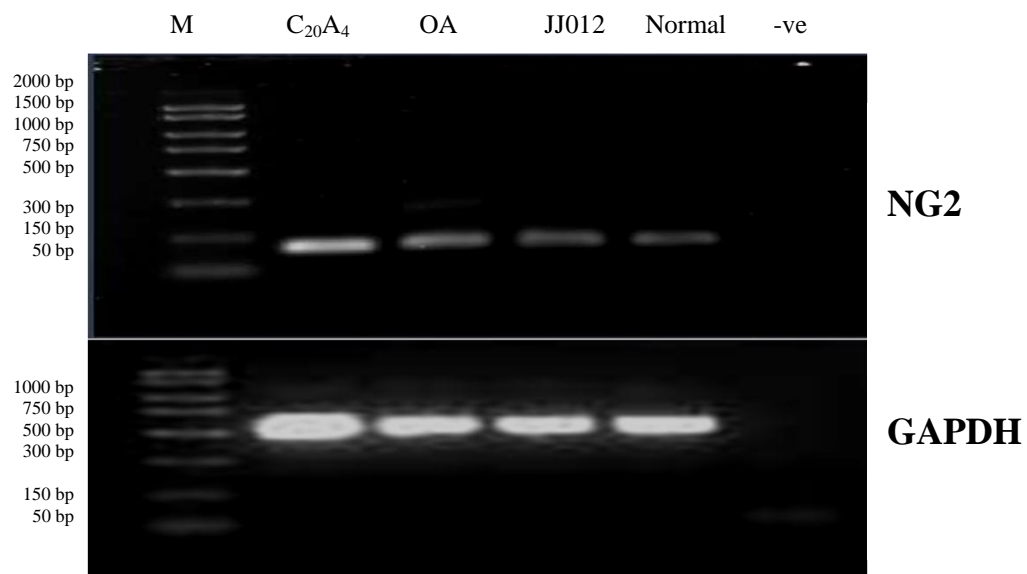


Figure 3.5: Representative image for RT- PCR reaction for NG2/CSPG4 expression. M represents PCR marker. GAPDH was used as a loading control. Samples used: C₂₀A₄ (passage 88), primary human osteoarthritic chondrocytes (OA) (sample number 14300), JJ012 (passage 16) and primary normal human chondrocytes (normal) (sample number 14282). 113 and 431bp were the product sizes of NG2 and GAPDH respectively.

3.2.2 Protein expression by western blotting

Having established that NG2/CSPG4 is expressed at mRNA level, I investigated the protein expression by western blotting.

A. Optimisation of protein extraction method

Three different protein extraction buffers were tried. The housekeeping gene (α -tubulin) was extracted by all buffers and detected in western blotting (figure 3.6). NG2/CSPG4 was seen in protein samples extracted by using RIPA buffer and sodium orthovanadate. However, no NG2/CSPG4 protein was observed by using SDS lysis buffer extraction (figure 3.6). After optimisation of the protein extraction method, RIPA buffer was the method chosen for all further extracts. Using both conditions for protein extraction (sodium orthovanadate and RIPA buffer) produce a good signal but RIPA was selected because it enables efficient cell lysis and avoiding protein degradation. RIPA Buffer also results in low background in immunoprecipitation and western blotting.

B. NG2/ CSPG4 expression in JJ012, C₂₀A₄ chondrocytes cell line and primary OA chondrocytes

NG2/CSPG4 is a trans-membrane proteoglycan receptor expressed as a high molecular weight protein >400kD (chondroitin sulphated form) and a core protein of 270-300kD (chondroitin sulphate free). Treatment of cells with chondroitinase ABC deglycosylates NG2/CSPG4 resulting in the expression of chondroitin sulphate free core protein.

Protein extracts were obtained from JJ012 cells, the C₂₀A₄ chondrocytes cell line and primary OA chondrocytes expanded *in vitro*. Table 3.3 contains all sample details used in these experiments. Western blotting was carried out using two anti-NG2 antibodies: firstly (LHM-

2) antibody and secondly the NG2 antibody clone (9.2.27). Using (LHM-2) antibody, results showed that NG2/CSPG4 was expressed as a core protein of a molecular weight around 270kD in JJ012 and chondrocyte cell line with and without chondroitinase ABC pre-treatment (figure 3.7 (a) and (b)). However, in protein extracts from OA chondrocytes, three different forms of NG2/CSPG4 were observed: a faint band at 270kD, a smear component of a molecular weight greater than 400kD and another faint band at around 117kD. When OA chondrocytes were pre-treated with chondroitinase ABC, only 270kD was more intense and clear (figure 3.7a). However, not all human osteoarthritic samples used in this study expressed the low molecular weight NG2 (117kD) and only 6 out of 10 samples expressed that.

To confirm the previous data for the expression of low molecular weight form of NG2 in primary human OA samples, the second anti-NG2 antibody clone (9.2.27) was used. Results showed that NG2/CSPG4 was expressed by all cells studied. Western blotting of extracts from OA chondrocytes showed molecular weight forms of around 400, 270 and 55kD without chondroitinase treatment and four forms of 270, 238, 117 and 55kD following chondroitinase pre-treatment.

Table 3.3: The table below shows the details of human OA samples used in western blotting for NG2/CSPG4 expression.

Sample	Age	Sex	OA grade	Number of days in culture
14300	64 years	Female	I-II	14
14301	50 years	Female	Not known	11
14308	62 years	Female	I-II	13
14316	73 years	Male	I-II	13
14320	75 years	Male	Not known	12
14348	75 years	Female	I-II	14
14349	73 years	Male	Not known	10
14355	78 years	Female	I-III	10
14358	79 years	Female	Not known	13
14363	70 years	Female	Not known	15

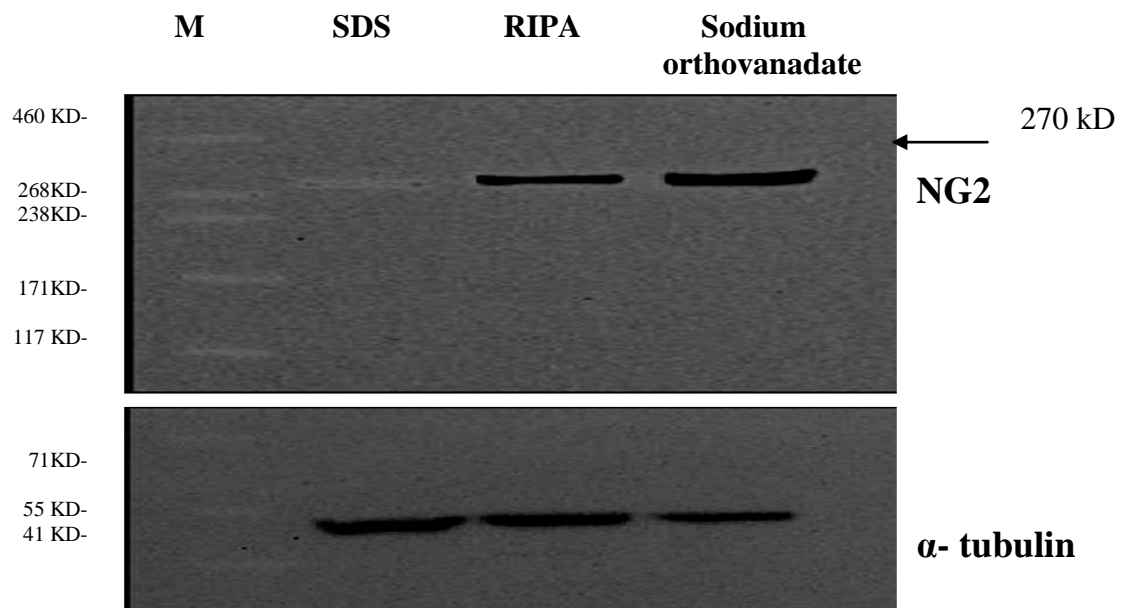
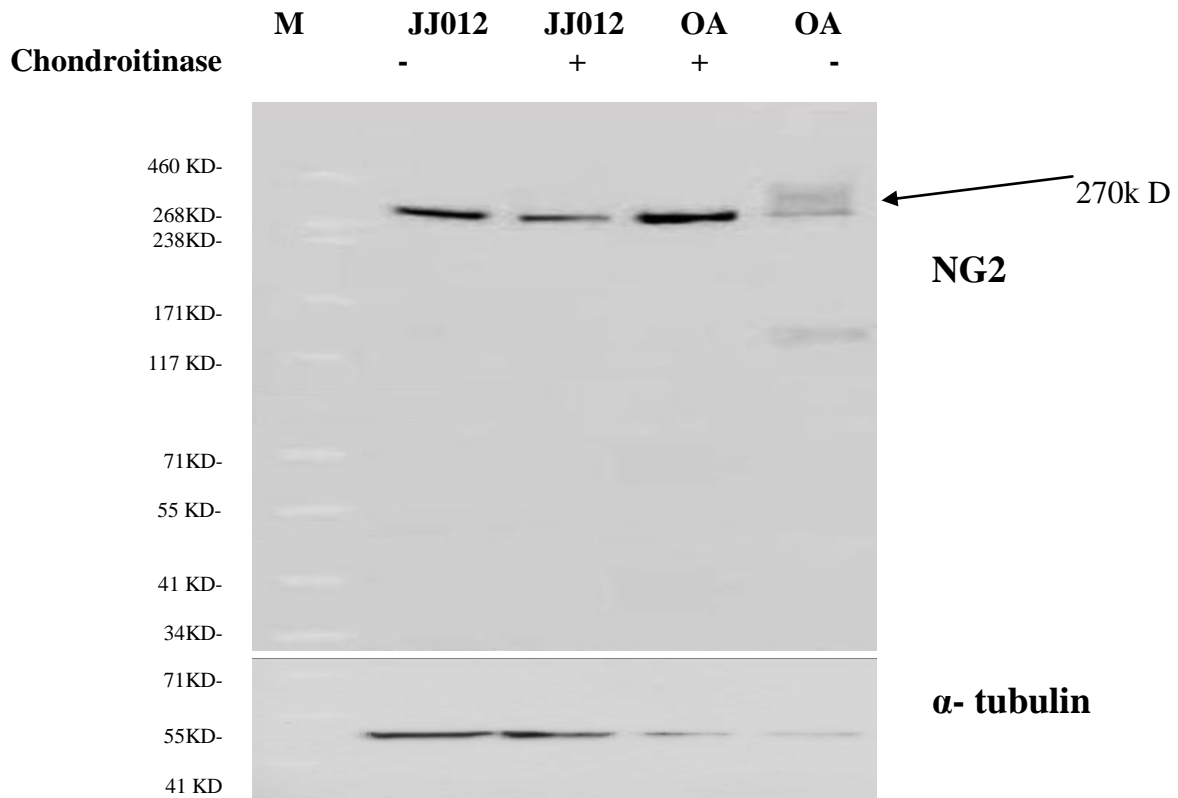


Figure3.6: Western blotting for NG2/CSPG4 expression in JJ012 cell line using different extraction methods. Cells were extracted using three different lysis buffers. Hi mark pre-stained, High Molecular Weight Protein Standard (m) was run on gel. Each lane represents cell extracts using one extraction method as labelled. Cells used were at passage 12.

(a)



(b)

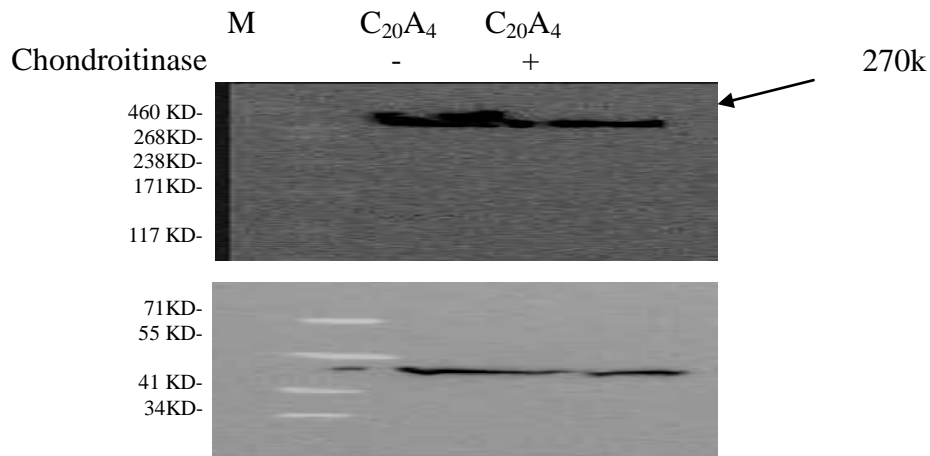


Figure 3.7: Western blotting for NG2/CSPG4 expression with (+) and without (-) chondroitinase ABC pre-treatment using (LHM-2) anti-NG2 antibody. (a) Represents western blotting images for JJ012 (n=3) cells and human OA chondrocytes (n=10) and (b) for C₂₀A₄ (n=3). Hi mark pre-stained High Molecular Weight Protein Standard (M) was run on gel. Samples used were: JJ012 (passage 12), human OA chondrocytes (14300) and C₂₀A₄ (passage 87). α - tubulin was the housekeeping gene use.

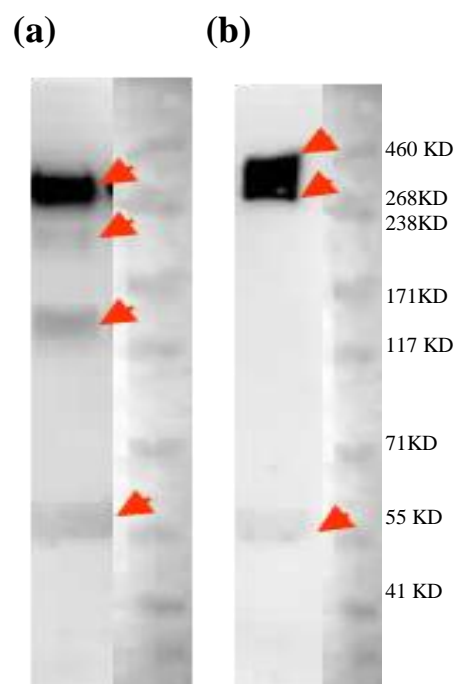


Figure3.8: Western blotting for NG2/CSPG4 expression with (+) and without (-) chondroitinase ABC pre-treatment using clone (9.2.27) antibody in human OA chondrocytes. (a) Represents western blotting images with chondroitinase ABC pre-treatment and (b) without chondroitinase ABC pre-treatment. Hi mark pre-stained High Molecular Weight Protein Standard (M) was run on gel. Human OA chondrocytes (14300) were used. α -tubulin was the housekeeping gene used (52kD).

3.2.3 NG2/CSPG4 surface protein expression by flow cytometry

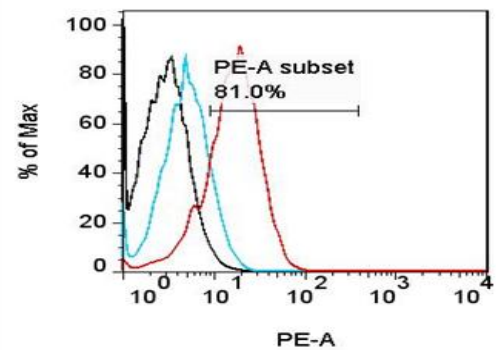
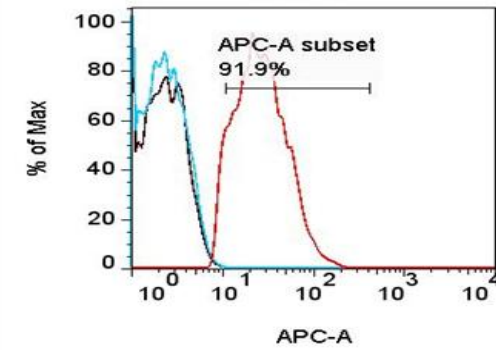
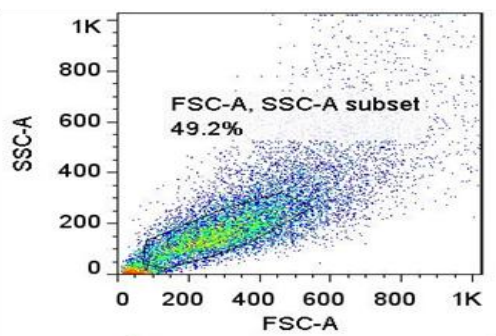
Flow cytometry was performed to show the percentage of cells expressing NG2/CSPG4 on the cell surface in JJ012, OA chondrocytes, chondrocyte cell line. Beta- 1-Integrin (CD29) was used as a positive control, which is expressed on the cell surface of all these cells. Isotype controls for both NG2/CSPG4 and CD29 were used. Data were acquired and analysed in a viable cell gate using Flow Jo software. Statistical analysis was carried out using One-way ANOVA.

Results showed that there was no difference in the percentages of positive cells for NG2/CSPG4 between JJ012 cells, OA chondrocytes and chondrocyte cell line. However, chondrocyte cell line had the highest value for MFI (79.93), followed by OA chondrocytes (50.83) and JJ012 cells (40.17) (figure 3.9 illustrates representative images for flow cytometry experiment and figure 3.10 shows the percentage of positive cells and MFI for the same experiments). There was a stastically significant and a very significant difference between C₂₀A₄ and OA and C₂₀A₄ and JJ012 respectively. The P values were 0.0259 and 0.0014 for the difference between C₂₀A₄ and OA and C₂₀A₄ and JJ012 respectively.

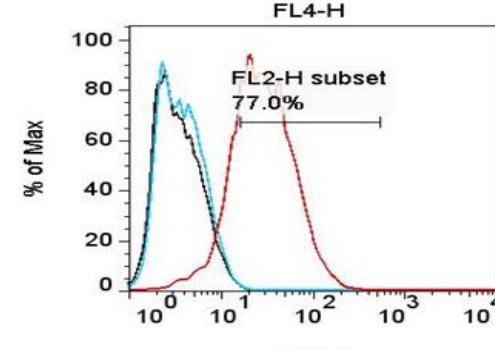
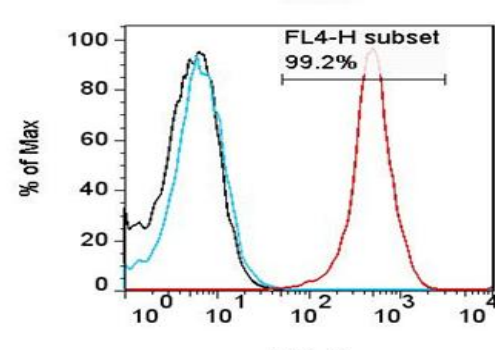
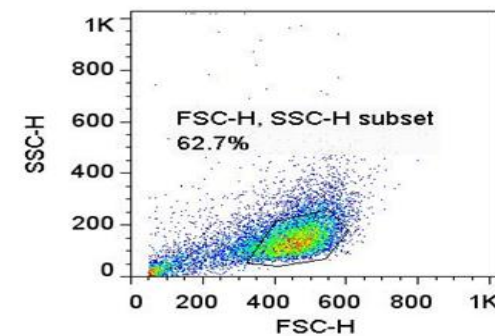
Table 3.4:

Details of human OA samples used in flow cytometry experiment to investigate NG2/CSPG4 expression.

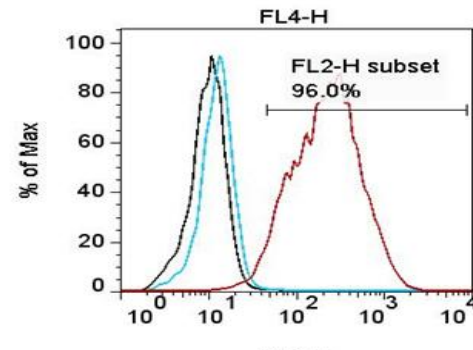
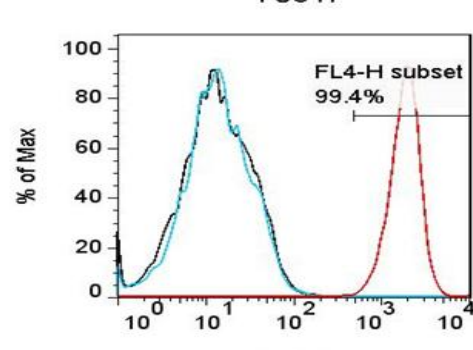
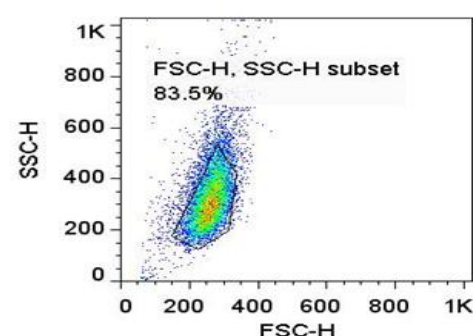
Sample number	Age	sex	OA grade	Number of days in culture
14309	82 years	Male	Not known	12 days
14316	68 years	Male	I-II	14 days
14324	65 years	Female	Not known	10 days
14325	81 years	Female	Not known	13 days
14333	64 years	Male	I-II	15 days
14355	78 years	Female	II-III	12 days



(a)



(b)



(c)

Figure 3.9: Representative image for flow cytometry results (histogram analysis) for NG2/CSPG4 expression. (a) In JJ012 cells (n=3) (b) in primary human OA chondrocytes (n=6) and (c) in C₂₀ A₄ (n=3). CD29 was used as a positive control. Isotype antibody controls for NG2 (PE-A or FL2-H) and CD29 (APC-A or FL4-H) were used. Samples were analysed using FACSCalibur (BD). No antibody was added in unstained sample. In each image three coloured graphs have been shown: red= anti-NG2 (LHM-2) antibody or anti-CD29 antibody (BD bioscience, UK), Black= unstained and blue represents the isotype control. Percentages of cells positive for both NG2 and CD29 were are shown in image presented.

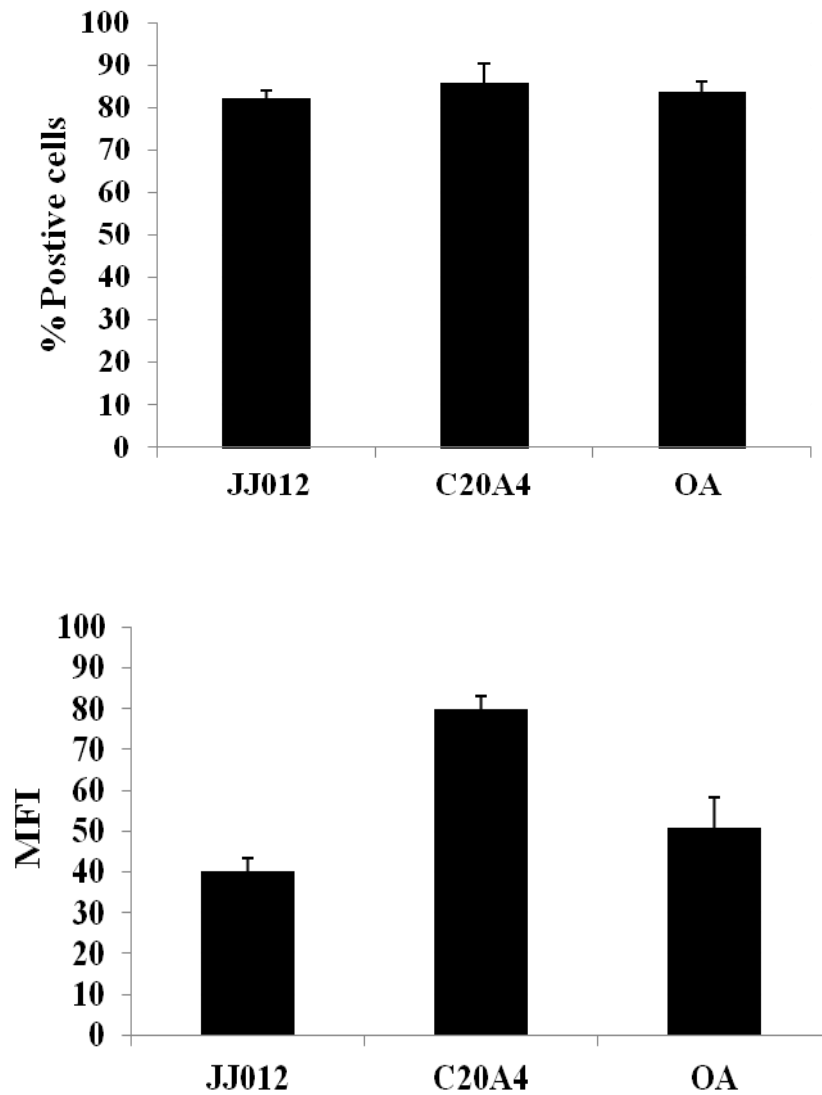


Figure 3.10: The graphs show the percentages of positive cells (a) and MFI (mean fluorescent intensity) (b) for NG2/CSPG4 expression in JJ012 (n=3), primary OA chondrocytes (n=6) and C₂₀A₄ (n=3) obtained by flow cytometry experiment. No difference in the % of positive cells between JJ012, primary OA chondrocytes and C₂₀A₄. C₂₀A₄ had the highest MFI (79.93), followed by primary OA chondrocytes (50.83) and JJ012 (40.17). There was a statistically significant and a very significant difference between C₂₀A₄ and OA and C₂₀A₄ and JJ012 respectively. The P values were 0.0259 and 0.0014 for the difference between C₂₀A₄ and OA and C₂₀A₄ and JJ012 respectively.

3.2.4 NG2/CSPG4 expression by indirect immunofluorescence

To demonstrate NG2/CSPG4 surface localisation on individual cells, indirect immunofluorescence was performed. NG2/CSPG4 expression was examined in JJ012 cells, OA chondrocytes and chondrocytes cell line C₂₀A₄. Negative controls were obtained by omitting the primary antibody.

Results showed that there was different distribution of NG2 in the cells studied. NG2/CSPG4 was distributed all over the cell surface and formed projections from the cell surface in JJ012 cells (figure3.10). In OA chondrocytes, NG2/CSPG4 was shown to be distributed in a punctate pattern inside the cytoplasm with less towards the periphery of cells (figure3.11). However, both patterns of distribution (punctate pattern inside the cytoplasm and cell surface projections) were observed in chondrocyte cell line (figure3.12).

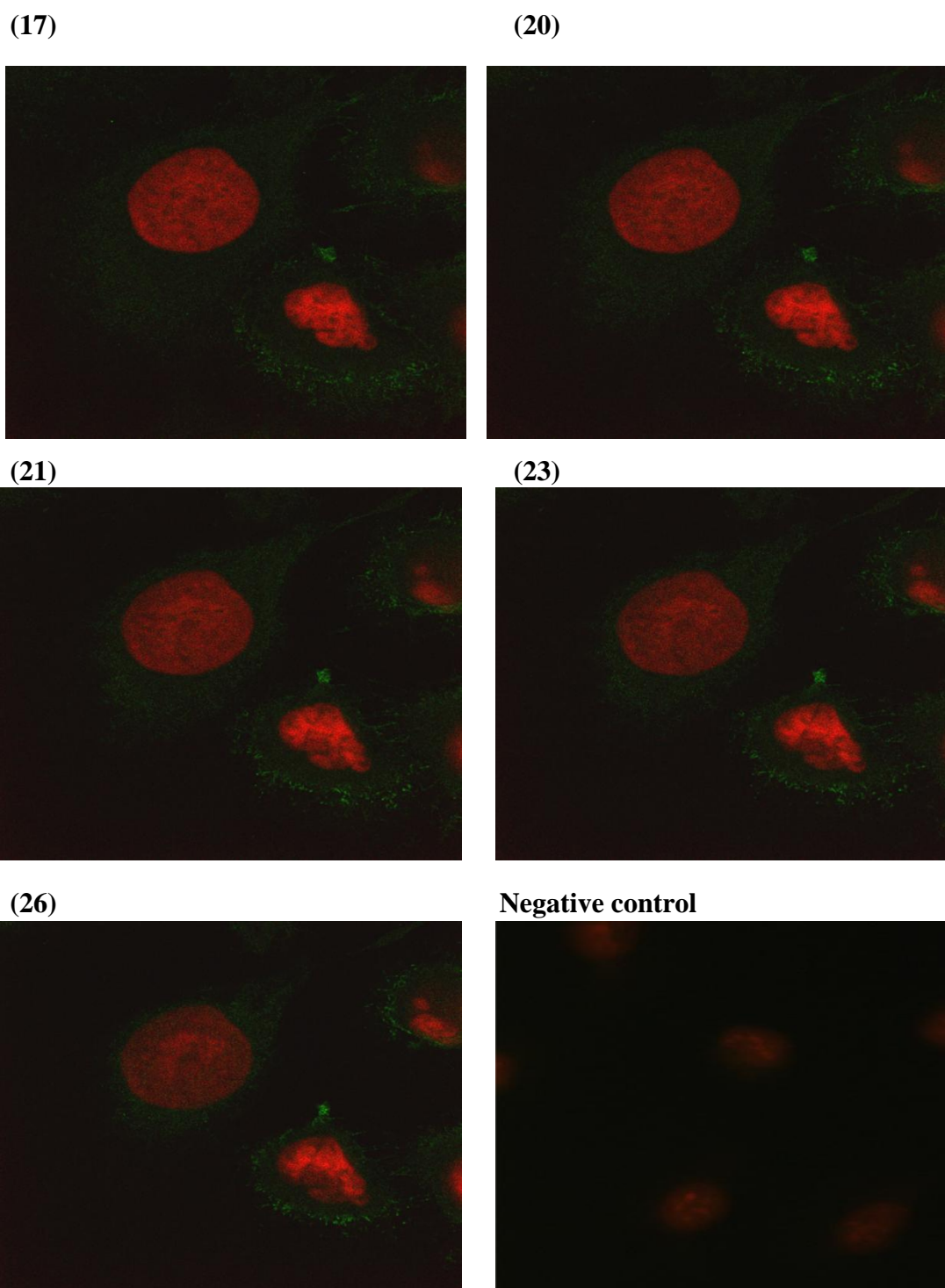


Figure 3.10: Representative image for indirect immunofluorescence for NG2/CSPG4 expression in JJ012 cell line (Passage 16). Images were taken using the confocal microscope at x100 objective. NG2/CSPG4 is distributed over the cell surface and forms projections. Images show represent different sections (section number written on the top of the image) of the stack. Negative controls were prepared by omitting of the primary antibody.

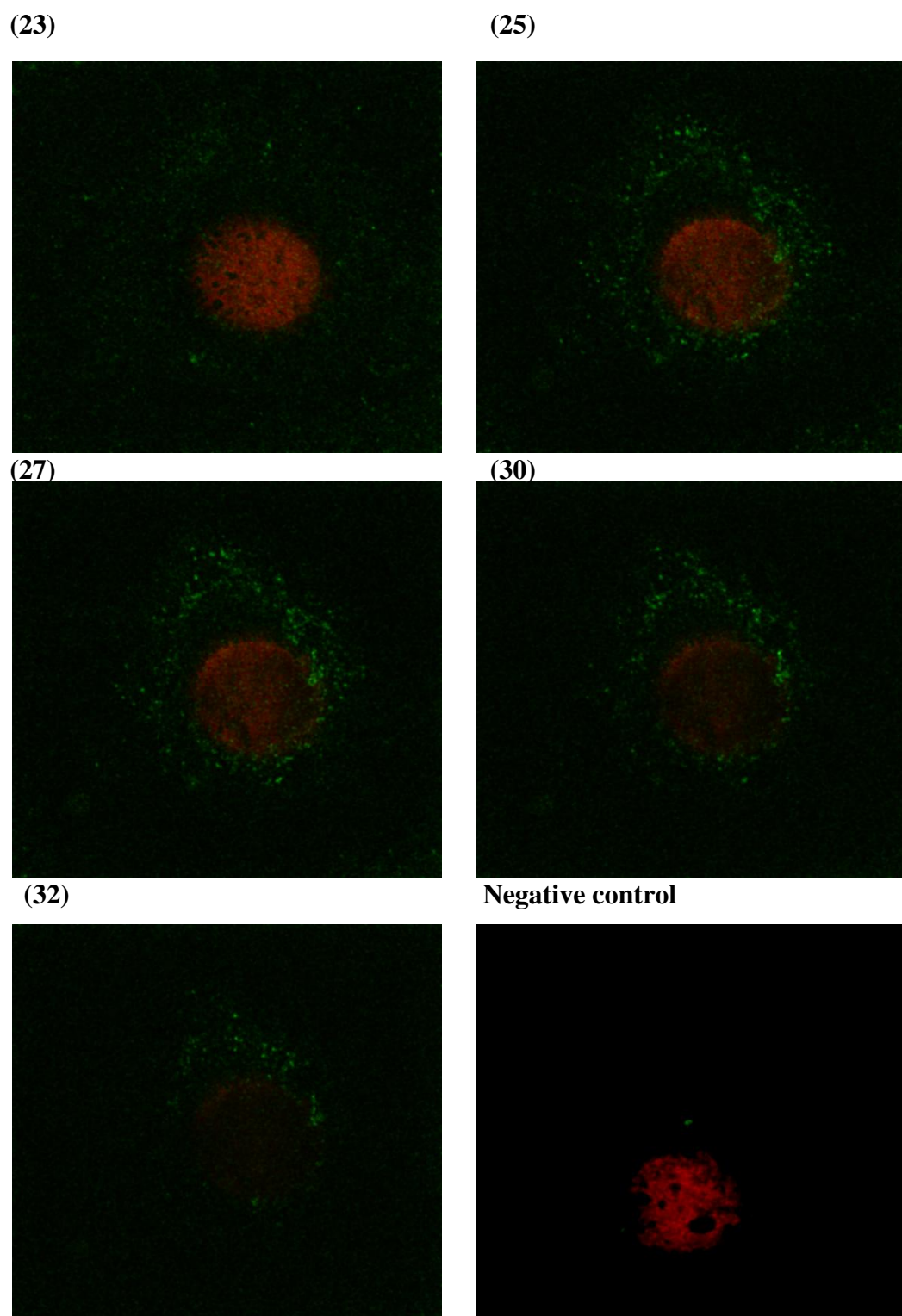


Figure3.11: Representative image for indirect immunofluorescence for NG2/CSPG4 expression in primary human OA chondrocytes (sample number 14300). Images were taken using the confocal microscope at x100 objective. NG2/CSPG4 is distributed in a punctate pattern inside the cytoplasm. Images shown represent different sections (section number written on the top of the image) of the stack. Negative control was prepared by omitting the primary antibody.

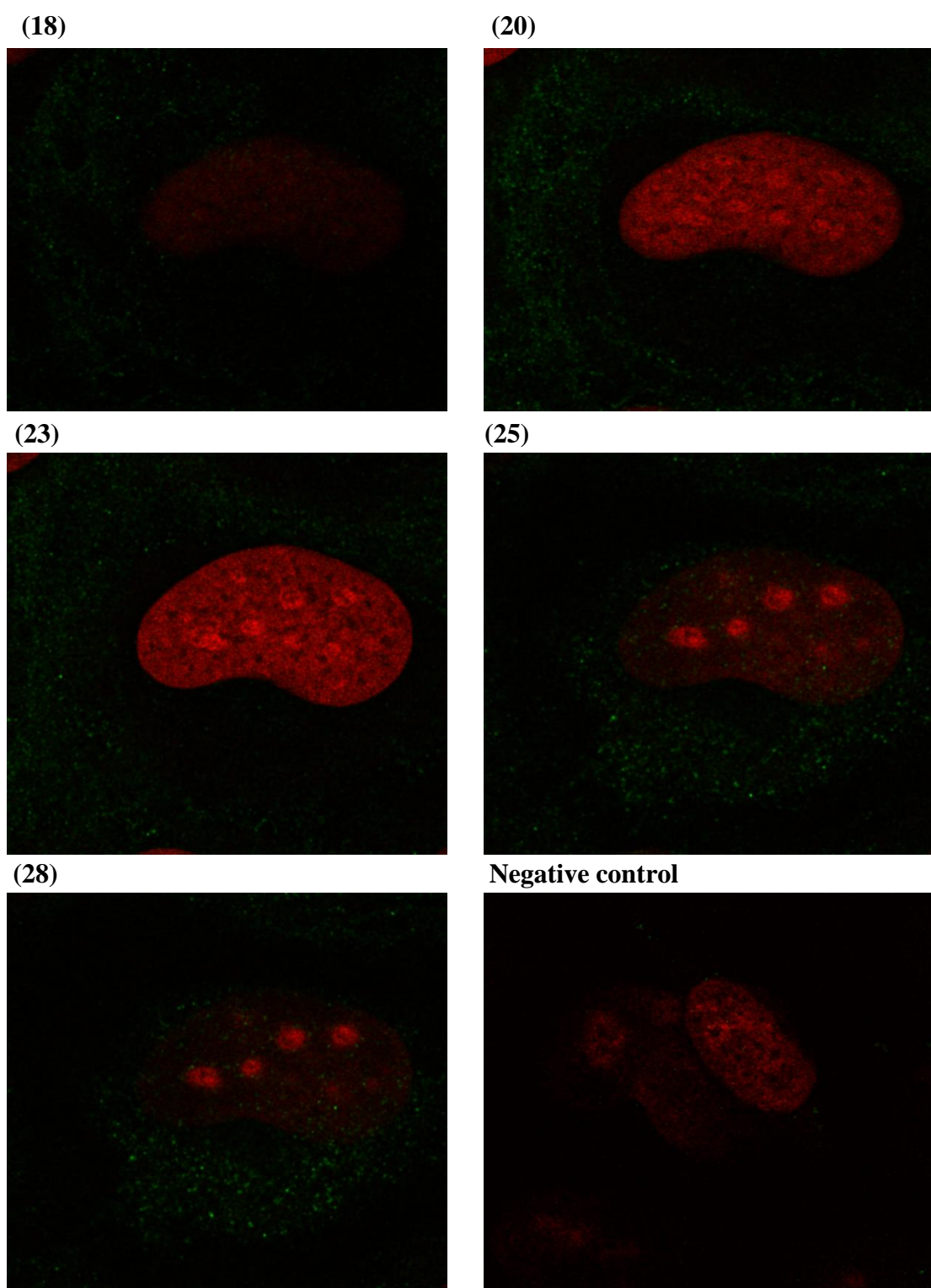


Figure 3.12: Representative image for indirect immunofluorescence for NG2/CSPG4 expression in chondrocytes cell line (passage 87). Images were taken using the confocal microscope at x100 objective. NG2/CSPG4 has two patterns of distribution (punctuate inside the cytoplasm and cell surface distribution). Images shown represent different sections (section number written on the top of the image) of the stack. Negative control was obtained by omitting the primary antibody.

3.2.5 NG2/CSPG4 sub-cellular localisation in primary human articular chondrocytes

In OA chondrocytes, NG2/CSPG4 was shown to be distributed in a punctate pattern in the cytoplasm with less expression towards the periphery of cells. Therefore, it was necessary to determine the NG2/CSPG4 sub-cellular localisation.

OA chondrocytes were grown in culture and double immunofluorescence was used to determine the interaction of NG2/CSPG4 with either endoplasmic reticulum(ER) or Golgi complex. The analysis of the data were carried out using Image J soft ware. CALM protocol 008 written by (Rolly Wiegand, 12.07.2011) was used. The following parameters were calculated:

- Pearson coefficient or R total (describes the correlation between the intensities of each channel in each pixel).
- The two threshold Manders percentages (tM1 and tM2) (attributes a value for each channel).
- The 2D histogram (all pixel intensities of one channel plotted on the X axis versus the intensities of the same pixels of the other channels along the Y axis).

Results showed that, in OA chondrocytes, a significant fraction NG2/CSPG2 is co-localised with both ER and Golgi complex (Figure 3.13). Details of the co-localisation analysis are demonstrated in the table 3.5.

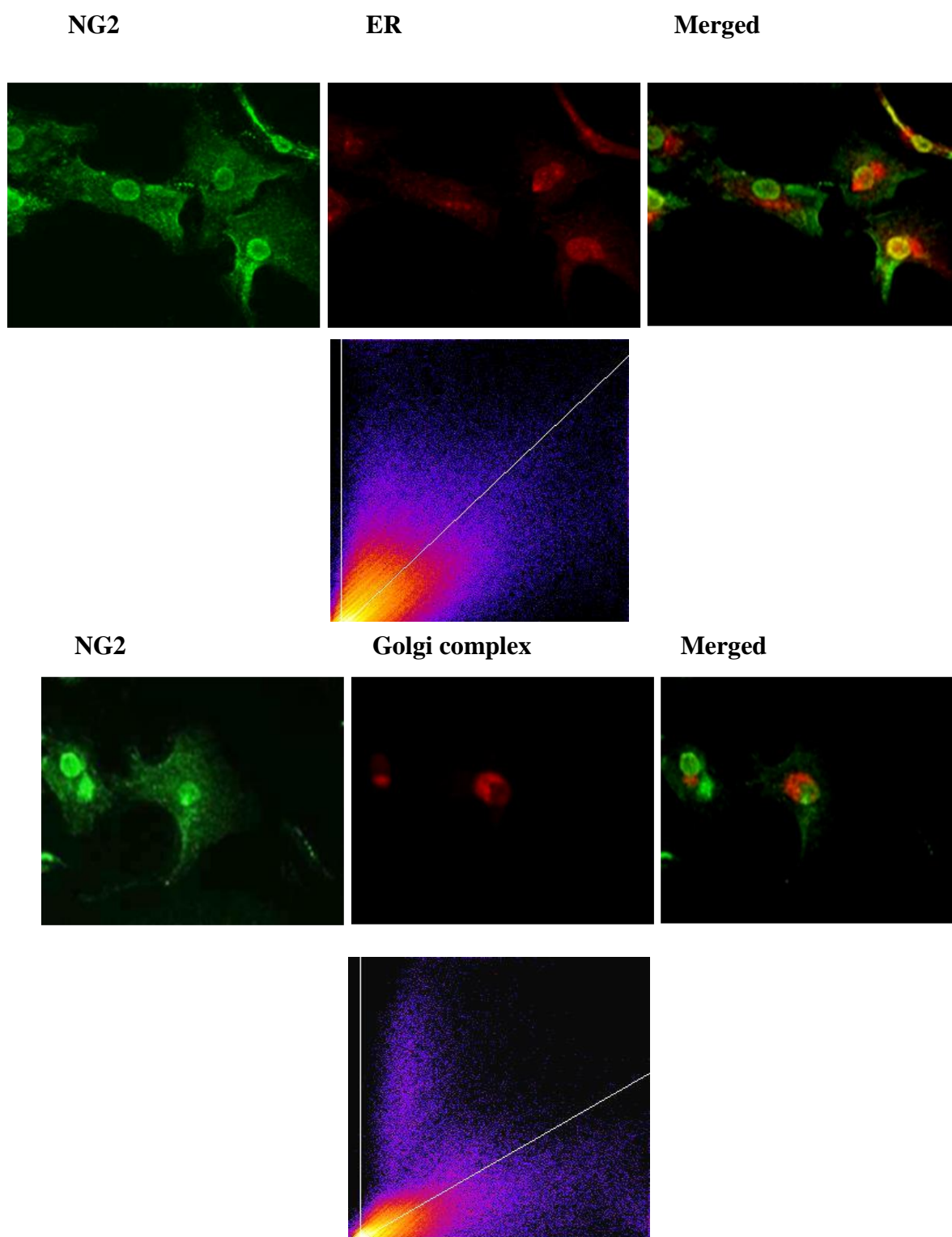


Figure 3.13: Representative images (n=3) for double immunofluorescence for NG2/CSPG4 sub-cellular localisation in OA chondrocytes. Images were taken using the confocal microscope Zeiss LSM 510 at x63objective (1pixel = 0.14um). The scatter plot for both (NG2 and either ER or Golgi associations) were shown. The scatter plot for both (NG2 and either ER or Golgi complex), which shows all pixels in the image as intensity channel 1 versus intensity of channel 2 also presented under the panel of images for each (NG2 and ER, NG2 and Golgi complex).

Table 3.5: The table below shows the details of the co-localisation analysis for double immunofluorescence of NG2/CSPG4 and either ER or Golgi complex. Results of n=3

Double immunofluorescence	Pearson coefficient	Threshold Manders(tM1)	Threshold Manders (tM2)
NG2 and ER	0.72	81%	89 %
NG2 and Golgi complex	0.59	64%	87%

3.2.6 NG2/CSPG4 Immunoprecipitation

The aim of this experiment was to investigate the association of NG2/CSPG4 with other molecules in both JJ012 and OA chondrocytes. Protein extracts were obtained from both JJ012 cells and OA chondrocytes. Samples were immunoprecipitated using rabbit polyclonal anti-NG2 antibody, run on western blotting gel, and then either the gel or the membrane was stained with comassie blue staining to look for the associated proteins. The experiment was carried out on n=1 sample due to time constraints.

Results showed that there were 6 associated bands with NG2/CSPG4 in OA chondrocytes, while no associated bands observed with NG2/CSPG4 in JJ012 cells. Figure (3.14) shows the results of this experiment.

The bands associated with NG2/CSPG4 in OA chondrocytes were identified by Mass Spectrometry. The bands were digested with trypsin and peptide finger print analysis was carried out using MALDI TOF Mass Spectrometry machine. The Voyager (Applied Bio system, UK) data was analysed using Data Explorer software and the proposed mass hits were searched against the Swiss Port database.

Result of the analysis is shown in table 3.8. It showed that NG2/CSPG4 is associated with collagen VI ($\alpha 1$ and 2) and Thrompoeitin in OA chondrocytes (where the %coverage was above the significance score). Although the analysis of the other bands showed that the % coverage was below the significance score, the possible associated proteins were stated based on the molecular weight or the presence of two dominant peaks in the protein spectrum.

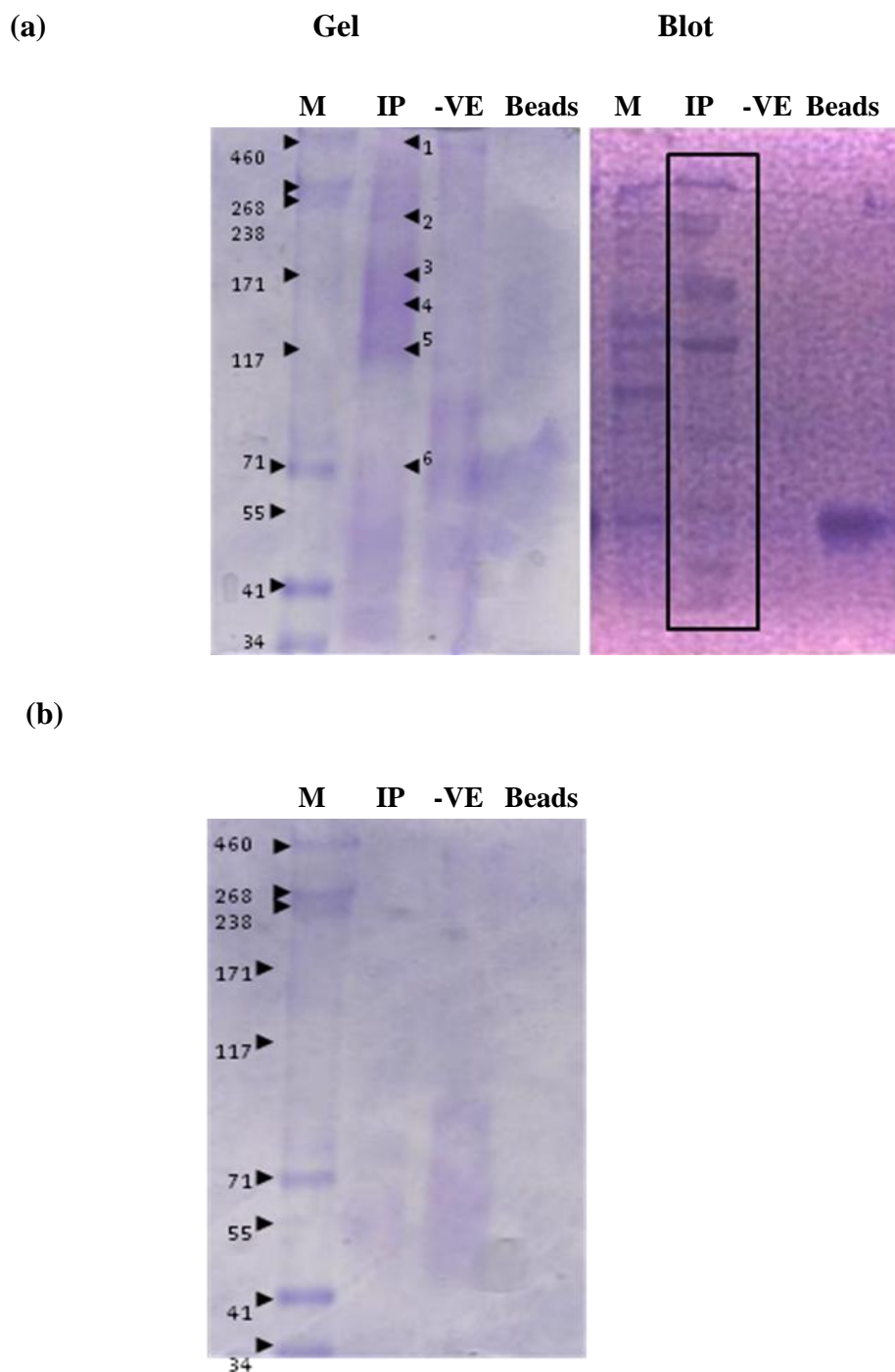


Figure 3.14: Coomassie Blue staining of the protein gel and PVDF membrane for the immunoprecipitated protein samples from OA chondrocytes (a) and Coomassie Blue staining on the protein gel only for JJ012 cells (b). For OA chondrocytes, the area marked with the box and labelled 1-6 represents the associated proteins. These bands were identified following mass spectrometry protocol using MALDI TOF MS machine (See table 3.8 below) M represents the high molecular weight marker, IP (immunoprecipitation protein sample), -ve(negative control) and beads(beads only control).

Table 3.6: The table below shows the details of the protein summary of the bands associated with NG2/CSPG4 in OA chondrocytes. The bands with a coverage percentage above the significance score are shown in bold.

Band Number	Protein identified	Molecular weight	Accession number	MALDI peptides	% Coverage	Above or below the significant score
1	• Uncharacterized protein FLJ46347	53869	Q6ZRH9.1	10	59	Above
	• Matrix remodelling - associated protein 5.	314065	Q9NR99.3	10	36	Below
	• Collagen alpha-1(V) chain	184131	P20908.3	5	31	Below
2	Collagen alpha-2(VI) chain	109709	P12110	16	43	Below
3	Collagen VI alpha-1	109602	P12109	18	80	Above
4	Collagen VI alpha-2	109709	P12110	22	103	Above
5	Thrompoeitin	38027	P40225.1	9	66	Above
6	Putative Fidgetin-like protein 2	67189	A6NMB9	6	44	Below

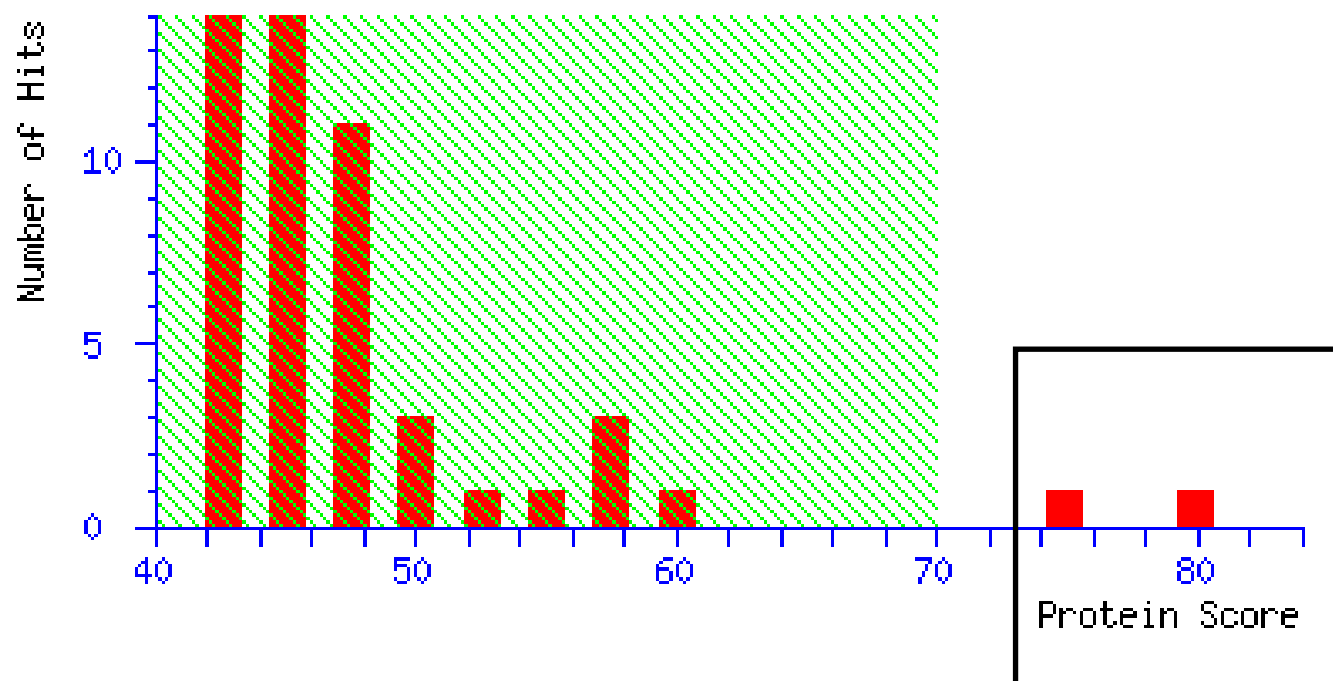


Figure 3.15: This graph is an example of the protein analysis results. It represents the analysis of the band three, which is Collagen VI alpha-1. The protein score achieved was 80% (represented by the highlighted black box), which is higher than the significant score (represented by the shaded green box in the graph). The Y -axis represents the percentage of the protein coverage, where in this graph, shows that it is higher than the significant score.

3.3 NG2/CSPG4 expression in primary human chondrosarcoma samples

Primary human chondrosarcoma cells were extracted from two human chondrosarcoma samples obtained from patients undergoing surgical treatment for chondrosarcoma. RNA and protein extracts were obtained from these two human chondrosarcoma samples and used to investigate NG2/CSPG4 expression by RT-PCR, western blotting and flow cytometry. A limited number of samples were used in these experiments because it was difficult to obtain fresh samples from patients.

3.3.1 NG2/CSPG4 gene expression by RT-PCR

Cells were extracted from two human chondrosarcoma samples and expanded invitro. RNA was extracted from two primary human chondrosarcoma samples and used to investigate NG2/CSPG4 expression by RT-PCR. Results showed that NG2/CSPG4 was expressed in both samples used (Figure 3.16).

There are 5 different types of chondrosarcoma: conventional primary central, conventional secondary peripheral, dedifferentiated, mesenchymal and clear cell chondrosarcoma. The dedifferentiated type of chondrosarcoma has two portions: cartilaginous portion, where cells express the cartilage matrix molecules and the non cartilaginous portion, where its cells express fibroblastic matrix components. Therefore, RT-PCR for two cartilage phenotype molecules (aggrecan and collagen II) was carried out to determine whether the cells expanded invitro from the primary human chondrosarcoma samples were obtained from cartilaginous or non cartilaginous portions of the tumours.

Results showed that there was an expression of aggrecan but not collagen II in sample (14307), which indicates that the cells were obtained from the non cartilaginous portion of the tumour. For sample (14292), both molecules (aggrecan and collagen II) were expressed by the cells expanded invitro, which means that cells were obtained from the cartilaginous portion of the tumour.

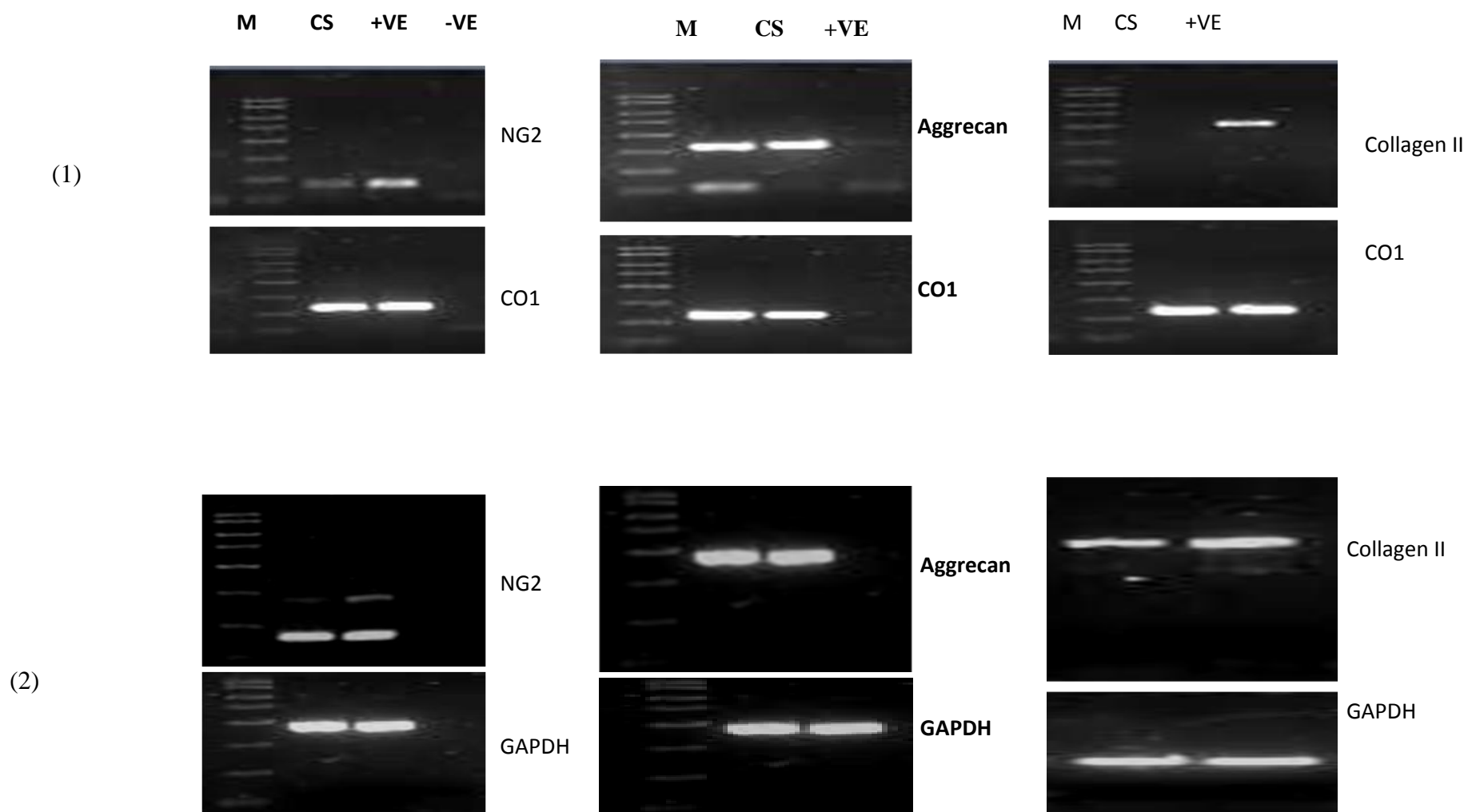


Figure 3.16: RT-PCR results for NG2/CSPG4, aggrecan and collagen II expression in primary human chondrosarcoma cells. Images present results for 14307 (1) and 14292 (2) chondrosarcoma samples. M, CS and +ve represent marker, primary human chondrosarcoma and positive control respectively. Details of all primers used in this experiment are illustrated in the Materials and Methods chapter.

3.3.2 NG2/CSPG4 protein expression by western blotting

Protein extract was obtained from one primary human chondrosarcoma sample (14292) only and used to investigate NG2/CSPG4 expression by western blotting. A very low protein concentration was gained from sample (14307), which was insufficient to use in western blotting.

Results showed that there was a clear band for NG2/CSPG4 expression in primary human chondrosarcoma sample (14292). NG2/CSPG4 was expressed as a chondroitin sulphate free molecule with and without chondroitinase treatment in this sample (Figure 3.17 a).

3.3.3 NG2/CSPG4 protein expression by flow cytometry

Cells were expanded *in vitro* and used to investigate NG2/CSPG4 expression by flow cytometry. Flow cytometry was carried out on one primary chondrosarcoma sample (14292). There were insufficient cells to perform flow cytometry on 14307 chondrosarcoma sample.

Although there was a clear band for NG2/CSPG4 in this sample tested (14292), there was no NG2/CSPG4 staining observed in cells extracted from this sample, expanded *in vitro* and tested to date (figure 3.17b).

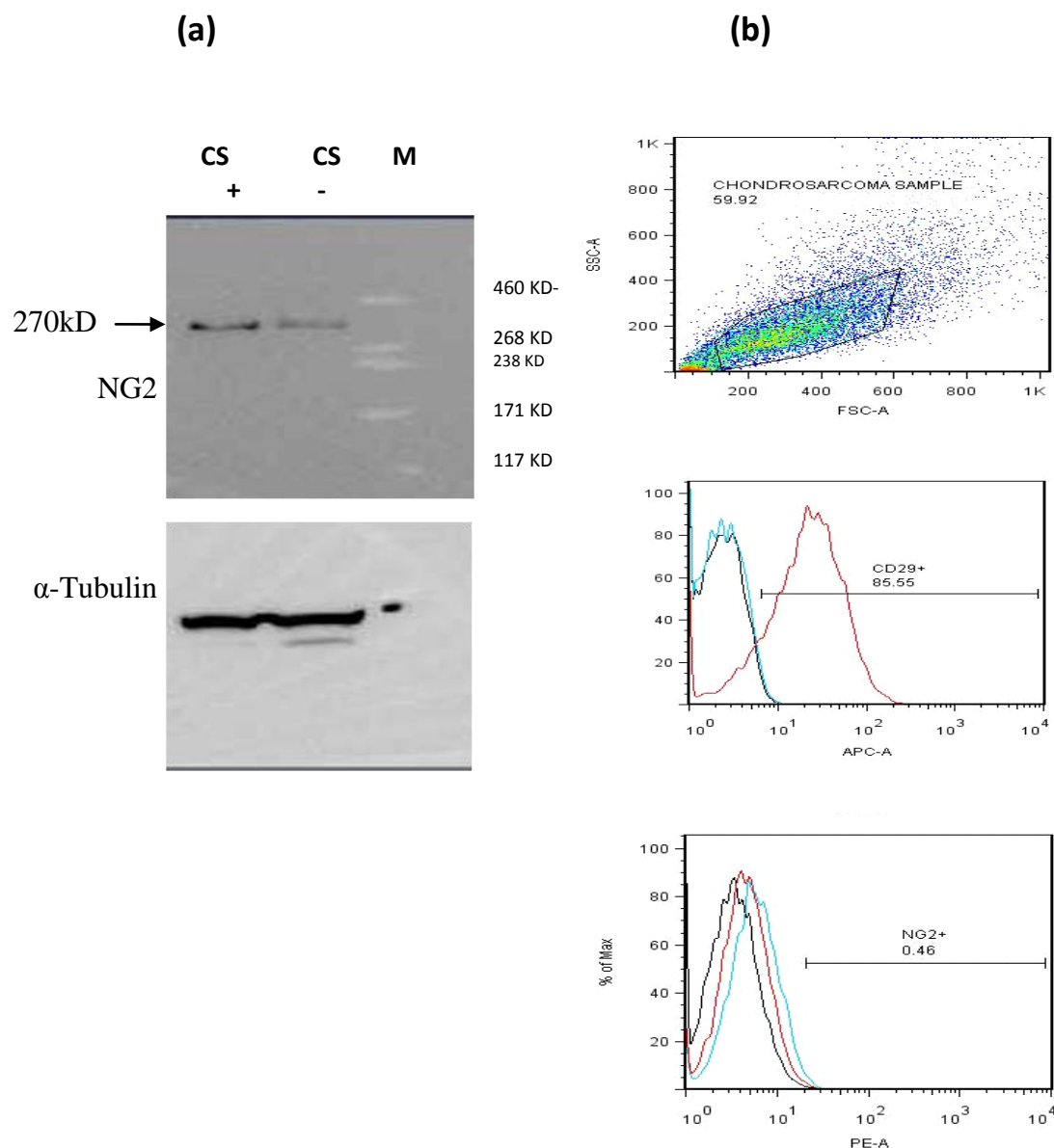


Figure 3.17: Western blotting (a) and flow cytometry (b) results for NG2/CSPG4 expression in primary human chondrosarcoma cells. Images in (a) represent the results for 14292 only with (+) and without (-ve) chondroitinase ABC treatment by western blotting, with M and CS representing marker and primary chondrosarcoma cells respectively. Images in (b) represent flow cytometry results for the same sample. CD29 was used as a positive control. Isotype antibody controls for NG2 (PE-A or FL2-H) and CD29 (APC-A or FL4-H) were used. Samples were analysed using FACSCalibur (BD). No antibody was added in unstained sample. In each image three coloured graphs are shown: red= anti-NG2 or anti-CD29 antibody, Black= unstained and blue represents the isotype control. Percentages of cells positive for both NG2 and CD29 were shown in images presented. Images shown represent results of n=1.

3.4 NG2/CSPG4 expression in different grades of human OA cartilage

3.4.1 NG2/CSPG4 gene expression in different OA scores samples from different patients

RNA was extracted from paraffin sections of different grades of OA samples. These samples were obtained from different patients who had undergone knee replacement surgery. Details of all samples used are shown in the table below (table 3.6 and 7). Samples were scored according to the method prescribed by Pritzker et al in 2006. Both grading and staging were determined for each sample and multiplied to achieve the score. Samples scored from 1-7 were considered as mild, from 8-18 as moderate and 16 and above as severe. The level of NG2/CSPG4 expression in each sample was normalised to the geometric mean of the three different internal control genes according to the paper published in 2002 by Vandesompele and his colleagues (Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control gene).

Results showed that there was no difference in the expression between different grades of OA (mild, moderate and severe (figure 3.18). There was an increased expression of NG2/CSPG4 in normal in comparison to OA samples; however, the difference was not statistically significant.

Table 3.7: The table below shows the details of human OA samples used in qPCR for NG2/CSPG4 expression in paraffin sections of different grades of OA from different patients

Sample	Age	Sex	OA grade	RNA quality
706	57 years	Male	Mild	1.8
727	65 years	Male	Mild	0.76
641	58 years	Female	Mild	1.2
693	70 years	Female	Mild	1.27
345	Not known	Not known	Mild	1.04
698	74 years	Female	Mild	0.91
610	60 years	Male	Moderate	1.49
740	75 years	Female	Moderate	1.5
622	77 years	Male	Moderate	1.3
347	Not known	Not known	Moderate	1.34
483	Not known	Not known	Moderate	1.4
621	Not known	Not known	Moderate	
718	68 years	Female	Severe	1.43
308	Not known	Not known	Severe	1.22
738	62 years	Female	Severe	2.32
360	Not known	Not known	Severe	1.3
631	Not known	Not known	Severe	1.2
306	Not known	Not known	Severe	1.93

Table 3.8: The table below shows the details of human normal samples used in qPCR for NG2/CSPG4 expression in paraffin sections of different grades of OA from different patients.

Sample	Age	Sex	RNA quality
14272	60	Male	1.06
14294	63	Female	1.13
14266	44	Male	0.3

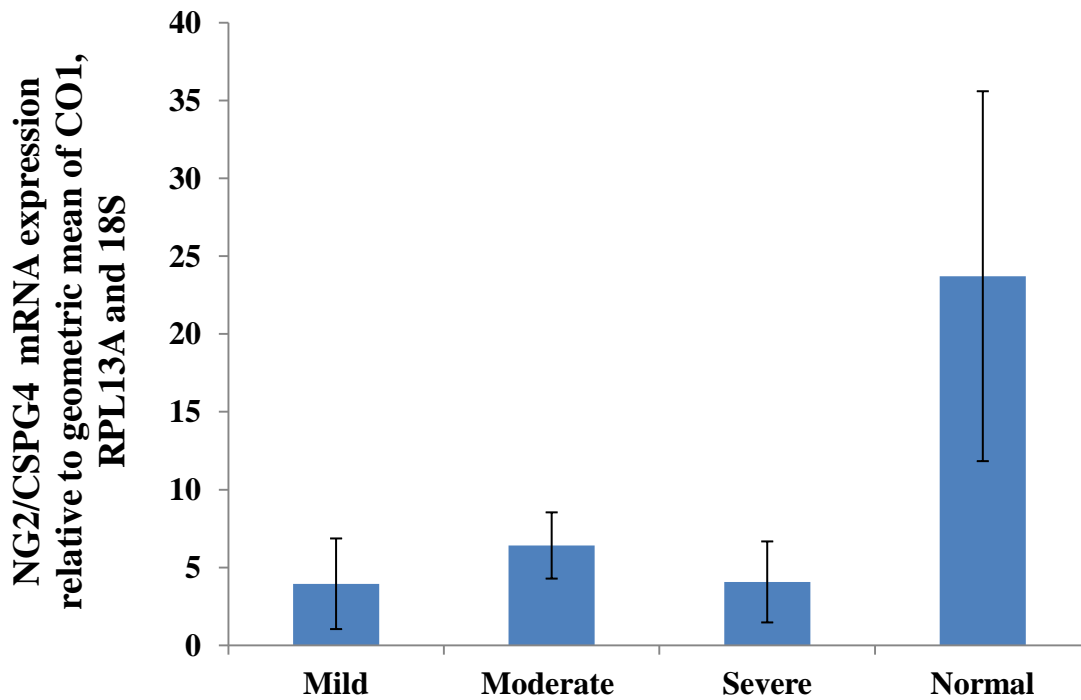


Figure 3.18: qPCR results for NG2/CSPG4 expression in RNA extracted from paraffin sections (OA (different grades) and normal). Values shown represent the mean \pm SEM. Results were normalised to geometric mean of the three housekeeping genes (RPLA13A, CO1 and 18S). There was no difference in the expression between different grades of OA (mild, moderate and severe). However, an increased NG2/CSPG4 mRNA expression was observed in normal cartilage samples versus OA; but the difference was not statistically significant.

3.5 Summary

NG2/CSPG4 gene expression was detectable in all cells tested by qPCR and RT-PCR. NG2/CSPG4 was expressed as a core protein of a molecular weight 270kD in JJ012 and (C₂₀A₄) chondrocyte cell line. In protein extracts from OA chondrocytes, three different forms of NG2/CSPG4 were observed: a faint band at 270kD, a smear component of a molecular weight >400kD and another faint band at 117kD. Following chondroitinase ABC treatment, only the 270kD band was evident. Western blotting of extracts from OA chondrocytes using anti-NG2 antibody (9.2.27) showed molecular weight forms of around 400, 270 and 55kD without chondroitinase treatment and four forms of 270 238, 117 and 55kD following chondroitinase pre-treatment. In flow cytometry, no difference in the % of positive cells between JJ012, primary OA chondrocytes and C₂₀A₄, however, C₂₀A₄ cells had the highest MFI (79.93), followed by primary OA chondrocytes (50.83) and JJ012 (40.17). NG2/CSPG4 was distributed all over the cell surface in JJ012 cells, while in OA chondrocytes, it was shown to be distributed in a punctate pattern in the cytoplasm with less towards the periphery. Both patterns of distribution were observed in the chondrocyte cell line. Frozen sections showed strong immunoreactivity of NG2/CSPG4 in human chondrosarcoma and OA cartilage.

In OA chondrocytes NG2/CSPG4 was present at the cell surface and within the cytoplasm where it was localised with the Golgi complex and ER. Immunoprecipitation and mass spectrometry data demonstrated associations between NG2/CSPG4 and both collagen VI and thrombopoietin in OA chondrocytes.

3.6 Discussion

3.6.1 NG2/CSPG4 expression in different types of chondrocytes used in this study

One of the aims of this study was to investigate the expression of NG2/CSPG4 in human chondrosarcoma, which has not yet been identified. In this study, I found that NG2/CSPG4 is expressed in human chondrosarcoma. This has been demonstrated in JJ012 chondrosarcoma cell line and in human chondrosarcoma samples. Strong immunoreactivity and mRNA NG2/CSPG4 expression level were shown in 7 human chondrosarcoma samples. More samples are required to accurately assess whether there is a difference in the level of mRNA expression between different grades of chondrosarcoma, however, due to the difficulty in getting these samples, this has not been done. For all other cells used in this study, there was no difference in the mRNA level of NG2/CSPG4 expression in JJ012, C₂₀A₄ chondrocyte cell line and OA chondrocytes by qPCR.

NG2/CSPG4 has significant similarities with human melanoma proteoglycans (HMPG), a molecule expressed by melanoma cells and by few adult human tissues (Nishiyama et al. 1991; Pluschke et al. 1996). The expression of NG2/CSPG4 in adult normal and osteoarthritic articular cartilage has also been identified (Midwood and Salter 1998). NG2/CSPG4 expression was found in normal and OA cartilage. However, its level of expression was down regulated in chondrocytes isolated from OA cartilage in comparison to normal cartilage (Midwood and Salter 1998). NG2/CSPG4 is also expressed in chemically induced chondrosarcoma in rat (Leger et al. 1994).

In this study, the level of NG2/CSPG4 expression was also evaluated by flow cytometry in JJ012, primary OA chondrocytes and C₂₀A₄ chondrocytes cell line. Results showed that there was no difference in the percentage of positive cells expressing NG2/CSPG4 between JJ012, primary OA chondrocytes and C₂₀A₄. However, C₂₀A₄ had the highest MFI (79.93), followed by primary OA chondrocytes (50.83) and JJ012 (40.17). There was a statistically significant and a very significant difference between C₂₀A₄ and OA and C₂₀A₄ and JJ012 respectively. This data indicates that, the number of cells expressing NG2/CSPG4 was the same between all cells used. However, the protein amount of NG2 expressed was different, with the highest being for C₂₀A₄ chondrocyte cell line, followed by OA chondrocytes and then JJ012. This may indicate more functional roles of NG2/CSPG4 in one cell type rather than others and highlight its existence as more important in chondrosarcoma in comparison to osteoarthritis.

NG2/CSPG4 expression was evaluated by flow cytometry in bone mesenchymal stromal cells (MSC) at different passages and results were compared with other MSC markers (CD73, CD105 and CD166), which are also expressed on the cell surface (Ilknur Kozanoglu et al 2009). Results showed that NG2/CSPG4 is a promising marker to study the MSC biology, as the percentage of NG2/CSPG4 positive cells was similar to the percentage of other MSC markers mentioned and there was no difference in NG2/CSPG4 expression between different passage cultures. Thus, it may be useful to study the NG2/CSPG4 expression further in different grades of chondrosarcoma by flow cytometry and compare the amount of NG2/CSPG4 protein expression.

Cells were extracted from two human chondrosarcoma samples and expanded invitro. There was a limitation to this study due to a limited number of samples obtained, as well as the

difficulty in maintaining the cells viability in culture after extraction. Results indicated that NG2/CSPG4 expression was detectable at mRNA level in both samples used (14292 and 14307). However, western blotting and flow cytometry were only performed on sample 14292, because there was not enough material to perform the same experiments on sample 14307. NG2/CSPG4 expression was expressed as a CS free molecule on cells obtained from sample 14292. The expression of NG2 in this human chondrosarcoma sample was similar to JJ012 cell line. However, there was no NG2/CSPG4 expression shown by flow cytometry. There is a possibility that cells used in the flow cytometry experiment were extracted from a dedifferentiated part of the tumour, while the protein extract was obtained from the differentiated part of the human chondrosarcoma sample. The dedifferentiated portion of chondrosarcoma has the histological features of a fibrosarcoma and is no longer cartilaginous in its structure.

The expression of NG2/CSPG4 in human chondrosarcoma raises the possibility that NG2/CSPG4 may have an important role in this tumour; especially as this tumour is regarded as chemo and radio resistant). Several studies explored the role NG2/CSPG4 in different types of tumours and found that NG2/CSPG4 is overexpressed by several tumour types that fail to respond to conventional chemotherapy, including glioblastomas, melanomas and some leukaemia (Schrappe et al. 1991; Behm et al. 1996; Li et al. 2003). This may indicate the importance to explore more about the expression and the functional roles of this receptor in human chondrosarcoma, one of these is its role in human chondrosarcoma chemo-resistance.

3.6.2 Biochemical analysis and cellular distribution of NG2/CSPG4 in human chondrocytes

NG2/CSPG4 is expressed as two different forms at the cell surface, both as a chondroitin sulphate (CS) rich (proteoglycan) PG and the unmodified core protein. NG2/CSPG4 was first identified as an epitope on the cell surface of neuronal and glial cells using the sequentially-absorbed rabbit anti-serum (Wilson et al. 1981). Then, Stallcup and his colleagues in 1983 were able to identify NG2/CSPG4 using monoclonal antibodies in the primary cultures of the rat brain by immunofluorescence technique (Stallcup 1981). NG2/CSPG4 was first identified as a chondroitin sulphate proteoglycan by immunoprecipitation, where it has been found that NG2/CSPG4 separated as two components on SDS-PAGE gel: a well defined 300kDa band and another high molecular weight smear (Stallcup et al. 1983). The two forms of NG2/CSPG4 seem to have different roles in different cells and conditions. Using solid phase binding assay in B49 rat glioma cells, it was found that NG2/CSPG4 can bind collagen VI in chondroitinase ABC treated and untreated cells (Burg et al. 1996). In addition, NG2/CSPG4 can bind to other ECM molecules, such as collagen II, V, laminin and tenascin. The binding domains of NG2/CSPG4 to collagens were similar to other collagen binding proteoglycans, such as decorin. However, the binding of NG2/CSPG4 to laminin and tenascin was receptor specific and was not inhibited by decorin. This is followed by another study, where Tillet and his colleagues found that the central non- globular domain of NG2 is the site for the interaction with type V and VI collagens (Tillet et al. 1997). The retention of collagen VI to the cell surface occurred normally in cells expressing chondroitin sulphate mutant NG2 (Stallcup 2002). In melanoma cell line, CS-GAG chains are responsible for the interaction of these cells with FN and modulate the adhesion property of these cells to fibronectin (Iida et al. 1992).

To investigate the biochemical characteristics of NG2/CSPG4 in human chondrosarcoma, and to compare that with primary OA chondrocytes and chondrocytes cell line, western blotting was performed. NG2/CSPG4 is expressed as a core protein only (CS free) in JJ012 cells and the chondrocyte cell line. However, the expression of NG2/CSPG4 in primary OA chondrocytes was different. Using two different anti-NG2 antibodies, I identified that NG2/CSPG4 is expressed as: 1) a core protein. 2) CS-PG and 3) further, as yet unidentified, bands at 238, 117 and 55kD. This different pattern of NG2/CSPG4 expression can serve different functions in different tissues.

The results shown in this study confirmed the previous data of NG2/CSPG4 expression in primary OA chondrocytes in our group (Midwood and Salter 1998). However, the expressions of low molecular weight forms of NG2 have not been shown before. Although the use of two different anti-NG2 antibodies was useful to confirm the presence of low molecular weight NG2 in OA chondrocytes, however, the sensitivity of the detection was different between the two antibodies. Further low molecular weight forms, at different levels, were detected with NG2 antibody clone (9.2.27) than (LHM-2). This can be attributed to recognition of different epitopes by different antibodies within the same receptor. This could represent proteolytic fragments of NG2/CSPG4 in OA chondrocytes.

Osteoarthritis (OA) is a progressive disease of the joints characterised by uncontrolled degradation of the extracellular matrix (Madry et al. 2011; Verma and Dalal 2011). In addition, it is well known that NG2/CSPG4 possesses sites for proteolytic activity, which lie in its juxta membrane one third of the ectodomain (known as Domain 2) (Nishiyama et al. 1995; Stallcup 2002). Previous studies highlighted that NG2/CSPG4 is susceptible to

proteolytic cleavage. Nishiyama and his colleagues in 1995 found that, in B49 rat cell lines (U251NG52 and 53), NG2 core protein is expressed as three forms (300, 290 and 275kD). These forms were originated from the same splice variant. However, subsequent experiments showed that the 300 core protein had undergone a proteolytic cleavage to yield the 275- and the 290-kDa forms. The activation of the protein kinase C stimulated the conversion of the 300 core protein form. Pulse-chase experiment proved that protein kinase C induced a transcription of gene encoding, a proteinase that is responsible for the cleavage of NG2. Although the exact enzyme and the site for NG2 cleavage was not clarified, metalloproteinase, such as collagenase and stromelysin, may have a role in this process, as NG2/CSPG4 contains cleavage sites for these two metalloproteinase. The interaction between NG2 and collagen type VI was found to protect the NG2 against cell surface proteolysis (Nishiyama et al. 1995).

The presence of such low molecular weight forms of NG2/CSPG4 in OA chondrocytes is a novel finding. Further studies are required in this aspect to explore the mechanism behind the generation of low molecular weight and identify the possible candidate responsible for the generation of these low molecular weight forms. It is also worth finding out whether NG2 can be identified in the media of OA cells.

NG2/CSPG4 is susceptible to a separate non shedding and shedding metalloproteinase-mediated effect. In 2003, in both rat and human peripheral nerves, all NG2 is saline soluble (shed), which represents the shed ectodomain NG2 (Morgenstern et al. 2003). Asher et al in 2005 showed that a high proportion of NG2 in adult rat spinal cord is saline soluble (shed). The high percentage of NG2 expression in injured cerebral cortex is mainly due to saline soluble (shed) rather than detergent-soluble (intact) form. The saline soluble migrates faster

on SDS gel rather than the detergent one. The conclusions were the rate of NG2 shedding is greater after CNS injury, which can have its impact on axon regeneration in the CNS. The use of metalloproteinase inhibitors prevent NG2 shedding in the oligodendrocytes progenitors, while the generation of the truncated form of NG2 was achieved TIMP-2 and TIMP-3 *in vivo*. It was not clear which metalloproteinase is really involved in NG2 shedding in ODP, however, more supportive data was in favour of the role of ADAMT 8 in this process (Asher et al. 2005).

Proteoglycans in general, are regarded as complex molecules. Proteoglycans are a group of glycoconjugates, composed from various core proteins, which are modified by post-translational modifications (PTM) with linear, anionic polysaccharide, glycosaminoglycans (GAGs). Different types of proteoglycans exist within different cells. The expression of the core protein as well as the GAGs chains is variable depending on the cell type as well as it is affected by the process of PTM, such as phosphorylation, N-linked or O-linked glycosylation. Furthermore, the expression of different forms of proteoglycans has its impact on the cellular functions and mutations of genes of these proteoglycans resulting in alteration of expression of the proteoglycans forms as well as the level of expression.

Neuroglycan C (NGC) is a transmembrane CSPG found predominately in the central nervous system (CNS). NGC changed its structure from a proteoglycan form to a non-proteoglycan form with the developmental stage of the cerebellum, whereas a small portion of NGC molecules existed in a non-proteoglycan form in the other areas of the mature CNS tissues. (Aono et al. 2004; Oohira et al. 2004). It was concluded from these studies that NGC gene expression is developmentally regulated. In addition, immunohistological and biochemical experiments suggested that NGC is involved in neuronal circuit formation in the central nervous system. This was further verified by finding that various NGC gene mutations in

mice caused the expression or glycosylation of NGC to be altered. The PTM of the lumican core protein affects the distribution of the integrin in melanoma RE cells (Brezillon et al. 2009).

Studies have shown that, even the interaction between different proteoglycans is dependent on the presence of a CS chain and more specifically on a specific subtype of this CS chain. CD44 is a good example to demonstrate this interaction. CD44 is one of the adhesion molecules that exist in two forms: CD44H or as a high molecular mass isoform. The interaction of CD44 with aggrecan derived from rat chondrosarcoma and bovine articular cartilage occurs via the CS chain (mainly that of CS-A), which has its important role at the inflammatory sites *in vivo* (Gagnon et al. 2000; Lewis et al. 2000; Wiater and Vale 2003). Also, the interaction of CD44 with serglycin modified is also CS-dependent (CS-A or CS-C or a mixture of both) (Shintani et al. 2006).

NG2/CSPG4 regulates the cellular response to growth factors (bFGF and PDGF-AA) (Goretzki et al. 1999). It was found that NG2 can regulate the activity of these factors, independent of the presence of GAG chain and the core protein of NG2/CSPG4, and appears to interact normally with both bFGF and PDGF-AA. In case of heparan sulphate proteoglycans, it interacts with bFGF via the glycosaminoglycan chains (Rapraeger 1995). Some of the other proteoglycans interact with their ligands independent of GAG chain. One of these examples is the interaction of CD44 with hyaluronan, betaglycan with TGF- β family and neuropilin interactions with VEGF and semaphorins at distinct sites on the protein (Schwarz and Ruhrberg 2010). Another example is the interaction of neuropilin-1 with vascular endothelial growth factor (VEGF) receptors and whose ligands include both VEGF and some semaphorins (Shintani et al. 2006). In endothelial cells in which HS substitution of

neuropilin is observed, signalling in response to VEGF is enhanced. In contrast, much of the vascular smooth muscle cell neuropilin-1 is chondroitin sulphate substituted and VEGF responses are depressed, which suggests that such forms of glycosylation of the receptor may significantly reduce the activity of this receptor.

Different patterns of NG2/CSPG4 expression in JJ012 and primary OA chondrocytes may be very useful to investigate the roles of NG2/CSPG4 interactions with other molecules in human chondrosarcoma and primary OA chondrocytes in the future. Good comparisons between different roles of NG2/CSPG4 in human OA cartilage and chondrosarcoma can lead to more useful studies towards more understanding of the disease process and the involvement of this receptor in both pathological conditions: chondrosarcoma and OA.

In this study, immunofluorescence results showed that NG2/CSPG4 was distributed all over the cell surface in JJ012 cells, while in OA chondrocytes, it was shown to be distributed in a punctate pattern in the cytoplasm with less towards the periphery. Both patterns of distribution were observed in the chondrocyte cell line. To further clarify the punctate distribution of NG2/CSPG4 in OA chondrocytes, and as previously illustrated for the role of Golgi apparatus and RER in the process of synthesis of NG2/CSPG4 (Stallcup et al. 1983; Thonar et al. 1983), double immunofluorescence was carried out using both NG2 and either Golgi complex or ER antibodies. Co-localisation analysis was carried out and showed that NG2 is co-localised with both Golgi and ER antibodies in OA cells.

As we discussed earlier, the process of synthesis of NG2/CSPG4 was studied by Stallcup and his colleagues in 1983 using pulse-chase experiments. It has been found that the core protein of NG2/CSPG4 is synthesised initially, with a molecular weight of 275kD, which contains the immature, high mannose oligosaccharide chains. After that, and within 60 minutes of the

synthesis process, this core protein undergoes modifications in the Golgi apparatus to produce the 300kD form core glycoprotein. During the same 60 minutes period, the chondroitin sulphate containing proteoglycans was also noticed. NG2/CSPG4 undergoes post translation modifications (Stallcup et al. 1983).

Earlier studies performed using cultured chondrocytes from the embryonic chick sterna and from the rat chondrosarcoma explained the post-translational modifications of the proteoglycans (Fellini et al. 1984; Geetha-Habib et al. 1984; Campbell and Schwartz 1988). One of these extensive post-translational processing of the core protein is the addition of N-linked oligosaccharides and xylose in the rough endoplasmic reticulum (RER) (Geetha-Habib et al. 1984; Hoffmann et al. 1984). This is followed by the addition of the remaining glycosaminoglycan chains and O-linked oligosaccharides and processing of the N-linked oligosaccharides in the Golgi apparatus (Thonar et al. 1983). Therefore, it appears that both Golgi apparatus and RER play important roles in the process of synthesis of NG2/CSPG4.

It has been found that the two organelles: Golgi complex and the closely related endoplasmic reticulum (ER) play an important role in the sorting of proteins to their various destinations. This is done by means of controlling various signals, inherent in the protein sequence, that dictate their ability to enter or avoid various destinations (Rothman and Wieland 1996).

Very early studies reported that there are changes in Golgi's status of the articular cartilage in different aspects: 1) secretion of extracellular matrix components (Martinez et al. 1977), 2) Drug effects (Moskalewski et al. 1975; Annefeld 1985; Stevens et al. 1985). 3) During development (Segawa et al. 1988), and 4) in clustered chondrocytes from a papain-induced cartilage defect in an experimental model (Scheck et al. 1975). This is followed by another

study which further supported previous conclusions. It showed that there was a disruption of the Golgi complex functions together with ER in surgically induced OA in rat cartilage in comparison to the normal articular cartilage (Kouri et al. 2002). This was demonstrated by a reduction in the staining intensities of both ER and Golgi complex as well as irregularity in the pattern of staining of both organelles. The irregularity of the pattern of staining of Golgi complex appeared as a compartmentalisation of the cytoplasm of OA chondrocytes, which was concomitant with the apoptotic nuclear changes. These observations were well correlated with the severity of OA progression and tissue damage in this OA model.

There may be a possibility that the disruption of the function of both organelles (RER and Golgi complex) may contribute to the defective processing (i.e. defective post-translational modifications) of NG2/CSPG4 in primary OA chondrocytes. or defective transport of NG2/CSPG4 to the cell surface, which in turn, collectively resulted in accumulation of NG2/CSPG4 in the cytoplasm of primary OA chondrocytes. This leads to the appearance of the punctate distribution of NG2/CSPG4 in these cells. An earlier study, to determine the main structure of CSPG and to identify the role of amino acid sequence for the attachment of GAG chains, showed that a significant intracellular proteolytic cleavage of the core protein does not occur during the post-translational modification of NG2/CSPG4, excluding the possibility of the intracellular proteolysis of the core protein. Therefore, the observation of the punctate distribution of NG2/CSPG4 in OA chondrocytes may not be attributed to the last mentioned point.

The post-translational modification (PTM) of the core protein can affect the function of the proteoglycan. The PMT of the lumican core protein, which interacts with melanoma cells, has been shown to have an important effect on integrin distribution within cells (Brezillon et al.

2009). Other examples like modification in the core protein of versican and decorin are important in colonic adenocarcinoma (Theocharis 2002). It has been found that there is a significant increase in the proportion of versican and decorin in cancer cells as well as a change in the type, length and the sulfation pattern of the GAG chain of these proteoglycans, which may have an important impact on the progression of human colonic adenocarcinoma. Further studies to explore the functional implications of this punctate distribution of NG2/CSPG4 in primary OA chondrocytes may lead to a better understanding of the OA process. The presence of this punctate expression of NG2/CSPG4 in OA chondrocytes and the deficiency of this punctate expression of NG2 in JJ012 cells may yield different roles of NG2/CSPG4 in both disease processes: chondrosarcoma and OA.

3.6.3 Association of NG2/CSPG4 with other molecules

Immunoprecipitation, in-gel digestion and mass spectrometry experiments were carried out to investigate the association of NG2/CSPG4 with other molecules in both JJ012 cells and primary OA chondrocytes. Interestingly, the results demonstrated that NG2/CSPG4 is associated with both collagen VI and thrombopoietin in primary OA chondrocytes, while no associated molecules were observed with NG2/CSPG4 in JJ012.

The association of NG2/CSPG4 with collagen VI has been investigated before in other cells. Since 1990, a study carried out by Stallcup and his colleagues demonstrated the interaction between NG2/CSPG4 and type VI collagen by immunoprecipitation, which was performed using different monoclonal antibodies against NG2/CSPG4 (Stallcup et al. 1990). In the same study, these results were further supported by performing double immunofluorescence experiment, which showed the co-localisation of NG2/CSPG4 and Collagen VI in different rat and human cell lines (Three rat cell lines: 1349, Bill, and B28 and two human cell lines:

IMR-90 and M21. Interestingly, it appears that, the loss of NG2/CSPG4 also affects Type VI collagen expression and distribution within the cells. The interaction between the two molecules appears to occur through protein-protein interaction rather than through the ionic interaction with the glycosaminoglycan chain (Stallcup et al. 1990).

Stallcup and his colleagues in 1993 found that cells lacking NG2/CSPG4 on cell surface, also failed to anchor type VI collagen, suggesting that NG2/CSPG4 functions as a cell surface receptor for anchoring type VI collagen to the cell surface (Nishiyama and Stallcup 1993). The functional significance of this NG2/CSPG4 and collagen VI interaction is further highlighted in subsequent research. It has been shown that the ability of transfected B28 glioma cells to migrate in response to collagen VI is increased, in comparison to their untransfected cells (Burg et al. 1997). Another study showed that NG2 is associated with type VI collagen and $\alpha 3\beta 1$ integrin in corneal mesenchyme cells and corneal fibroblasts during the different stages of stromal development of avian cornea. This highlights the possible functional implications of this interaction in cell migration, development and maintenance of corneal integrity (Doane et al. 1998). It appears that the collagen VI anchors to NG2/CSPG4 through its central domain, where it spans between the two extracellular globular domains of this receptor (Tillet et al. 1997). Therefore, cells lacking chondroitin sulfate chain can still interact and anchor type VI collagen (Burg et al. 1996; Tillet et al. 1997).

The role of both molecules: NG2 and collagen VI in the pathogenesis of OA has been investigated. It is well known that NG2/CSPG4 is a receptor for the extracellular protein and collagen VI, especially, as a ligand for NG2/CSPG4 (Stallcup and Huang 2008). A study done in our group showed that the adhesion of human normal articular chondrocytes was increased on collagen VI coated plates in the presence of NG2/CSPG4 stimulating antibodies,

while this effect is lost in the primary human OA chondrocytes. The loss of this activity indicates that NG2/CSPG4 may have an important role in the progression of OA (Midwood and Salter 2001). It is well known that, in OA process, there is a change in the distribution of collagen VI from its peri-cellular distribution to the interstitial spaces associated with up-regulation of this molecule. This is regarded as an attempt to compensate for the loss of the ECM environment (Hambach et al. 1998; Pullig et al. 1999; Soder et al. 2002). In another research study, immunofluorescence data showed that NG2/CSPG4 is down regulated as the OA tissue damage advances in eight patient-matched minimal- and advanced-OA samples (Nugent et al. 2009). Therefore, despite the fact that collagen VI is regarded as a cell surface ligand for NG2/CSPG4, it seems to be that both NG2 and collagen VI are opposing each other.

In case of Ulrich scleroatonic muscular dystrophy, an inherited disorder characterised by a collagen VI mutation, the loss of collagen VI is associated with down regulation of NG2 protein expression, while there is an increase in the NG2 mRNA expression. This is associated with up-regulation of tenascin C in the skeletal muscles. NG2/CSPG4 immunohistochemical and biochemical behaviour may be compromised owing to the absence of its physiological ligand. It appears that NG2 proteoglycan is considered an important receptor, mediating COL6-sarcolemma interactions and the disturbed relationship between NG2, and that collagen VI may be involved in the pathogenesis of UCMD muscle (Petrini et al. 2005; Nugent et al. 2009).

Another important finding is the association of NG2 with thrombopoietin in human OA chondrocytes. A study undertaken by Ceuninck and his colleagues in 2004 showed that thrombopoietin is one of the growth factors secreted by human OA chondrocytes into the

growth media. This was detected in stimulated chondrocytes with IL-1 β or TNF- α by protein microarrays (De Ceuninck et al. 2004). In addition, other proteins, growth factors and cytokines were also identified. Therefore, suggesting that all of these factors may have an important role in the pathogenesis of OA process. In addition, in synovial fibroblasts extracted from OA patients, it has been shown that IL-17 stimulated the secretion of thrombopoietin in a similar way that IL-1 β does as well as TNF (Honorati et al. 2004). In addition, spontaneous release was weak which was similar to effect of IL-1 β or IL-17

The association of NG2 with thrombopoietin in OA chondrocytes is a novel finding in this study. These data has not been illustrated before, not only in OA chondrocytes, but also in other cell types. This association may have a considerable role in OA process. Further studies are required in this aspect to explore further the importance of this association between NG2 and thrompoietin in OA pathogenesis

CHAPTER FOUR

Results

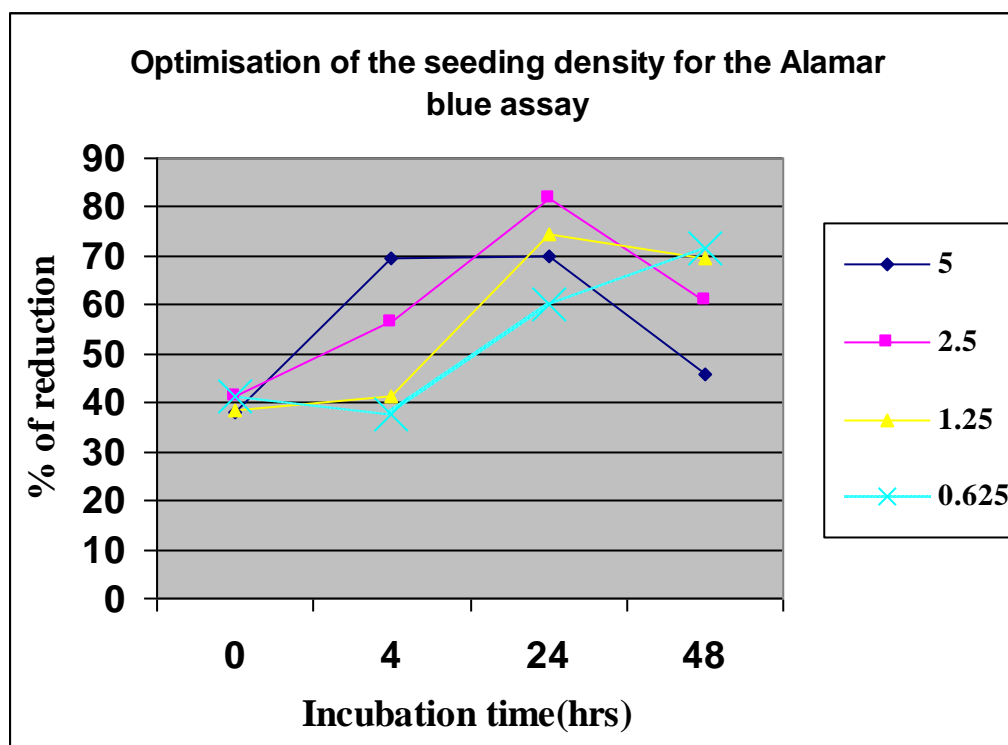
4.0 NG2/CSPG4 knockdown in JJ012 (grade II, chondrosarcoma cell line)

4.1 Optimisation of the puromycin killing curve for NG2/CSPG4 knockdown

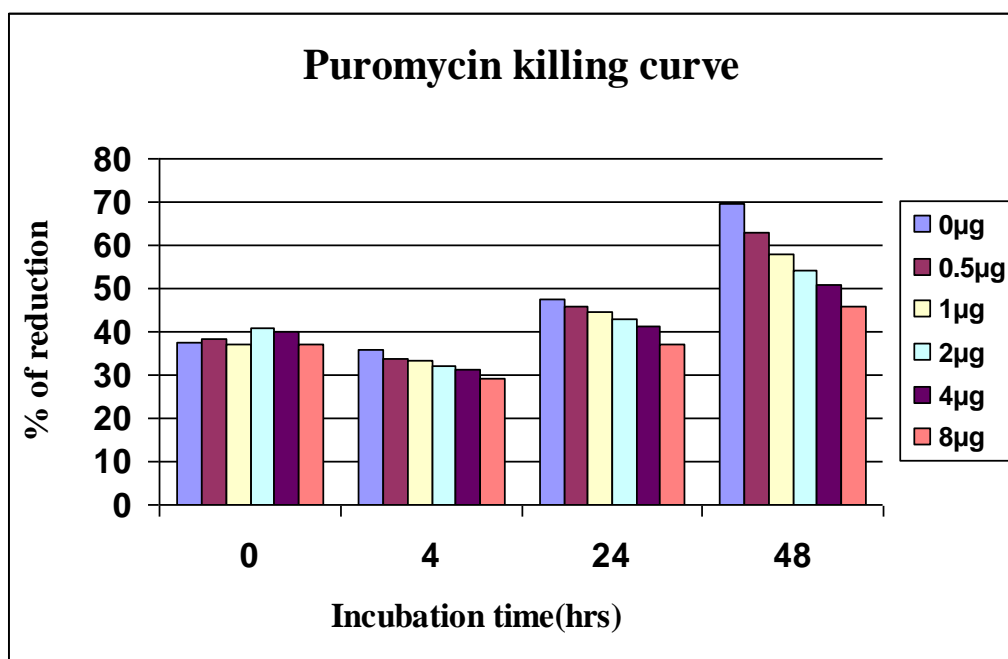
A puromycin killing curve was generated to determine the minimum amount of puromycin required to inhibit the growth or killing of non-transduced cells within 3 days and allow the selection of the resistant cells, in which lentiviral particles were incorporated. Alamar blue was used to establish the puromycin killing curve. Alamar Blue is a proven cell viability indicator that uses the natural reducing power of living cells to convert resazurin to the fluorescent molecule; resorufin (gives blue colour). Firstly, JJ012 NG2+ve cells were plated at different seeding densities (5, 2.5, 1.25 and 0.625×10^4 /well/100 μ l) to determine the best seeding density that shows a linear cellular growth within 3 days, using alamar blue assay.

Results showed that 0.625×10^4 /well/100 μ l is the best seeding density to use for establishing the puromycin killing curve (figure 4.1a). Next, JJ012 NG2 +ve cells were plated at 0.625×10^4 /well/100 μ l into the 96-well plates and grown for 24 hours in the incubator in the tissue culture room at 37°C. The next day, cells were incubated with puromycin at different concentrations 0.5, 1, 2, 4 and 8 μ g/ml and alamar blue was added to the wells. Readings (at 750 and 600 nm absorbance) were taken at 0, 24 and 48 hours after incubation. Alamar blue was preferable to establish the puromycin killing curve than MTT or CyQUANT® Cell Proliferation assay (used to assess the cell proliferation later on in this thesis) due to very low toxicity to cells, especially with the use of puromycin.

Results showed that there was a significant inhibition of cell growth with the addition of 1 and 2µg/ml of puromycin compared to untreated cells (0µg/ml graph of the results) (figure 4.1b). It is recommended to use the lowest possible concentration of puromycin to reduce its toxic effect; therefore, 1µg/ml of puromycin was used throughout the gene knockdown procedure.



(a)



(b)

Figure 4.1: Alamar blue assay results for establishing the puromycin killing curve: (a) optimisation of the seeding density, (b) puromycin killing curve. Puromycin was used as a selection indicator for the transduction process. Cells were seeded in 96 well plates and treated with puromycin at different concentrations (0.5, 1, 2 and 4 and 8µg). The effect of puromycin on the cell viability was assessed at 0, 24 and 48 hours after incubation.

4.2 Assessment of NG2/CSPG4 knockdown in JJ012 by quantitative qPCR

NG2/CSPG4 knockdown was carried out using the Mission TRC shRNA lentiviral transduction method as described in the Material and Method chapter. Five different constructs against NG2/CSPG4 (A, B, C, D and E) together with the negative (shRNA non-target) and the positive (shRNA GFP) controls were used in this experiment.

The gene knockdown efficiency was investigated by qPCR using the AB Biosystem 7500 thermal cycler. Specific primers for NG2 and CO1 (reference gene) were used in this experiment. First of all, we investigated the gene knockdown efficiency in RNA extracted from pooled cells (i.e. cells which are transduced with the highest amount of viral particles) of each construct used against NG2/CSPG4 (A, B, C, D and E). RNA extracted from negative (shRNA non-target) and the positive (shRNA GFP) controls were used in this experiment.

Results showed that B and D construct had the highest knockdown efficiency among all constructs used (i.e. the lowest gene expression max% normalized to JJ012 NG2+ve cells). The NG2/CSPG4 expression level was 12% and 10% for B and D constructs respectively (figure 4.2).

To achieve a higher efficiency for the two selected constructs (B and D), 5 resistant colonies (labelled from 1-5) were selected from each construct as described in the Materials and Methods chapter. After that, RNA was extracted and used to investigate NG2/CSPG4 expression level by qPCR.

Results showed that D1 and B3 have the highest knockdown efficiency among all resistant colonies used (i.e. the lowest gene expression max %). The NG2/CSPG4 expression level was 1.1% and 2.9% for D1 and B3 constructs respectively (figure 4.3).

4.3 Assessment of NG2/CSPG4 knockdown by western blotting

qPCR results showed that D1 and B3 constructs have the highest NG2/CSPG4 gene knockdown efficiency among all colonies selected. Western blotting was carried out to investigate the level of NG2/CSPG4 protein expression level in D1 and B3 cells. The procedure was performed using the antibody against NG2/CSPG4 (LHM-2). Protein extracts from GFP transduced cells and JJ012 NG2+ve cells were used as positive controls.

Results indicated that, although qPCR results showed that D1 cells has higher knockdown efficiency than from B3 cells at the gene level, there was no detectable level of NG2/CSPG4 protein expression in the protein extracts from B3 cells by western blotting. A band for NG2/CSPG4 protein was still observed in the protein extracts from D1 cells. Protein extracts from GFP and JJ012 NG2+ve cells showed a detectable level of NG2/CSPG4 protein expression (figure 4.3b).

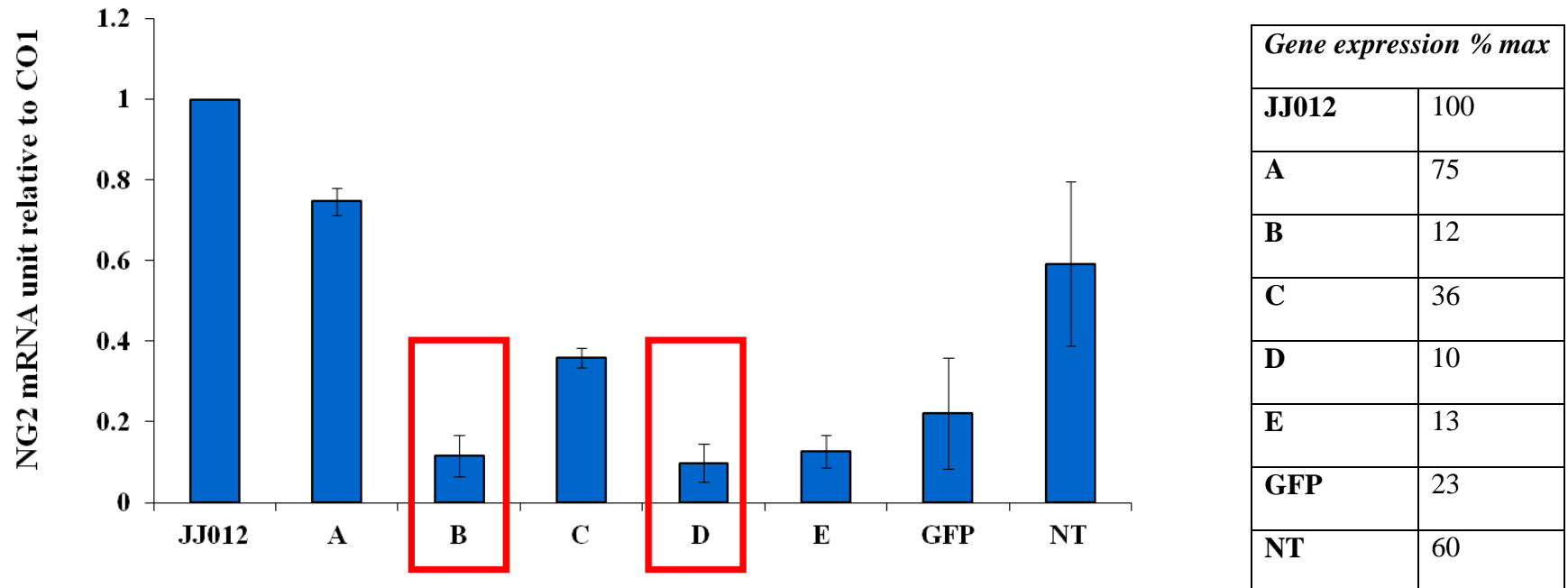
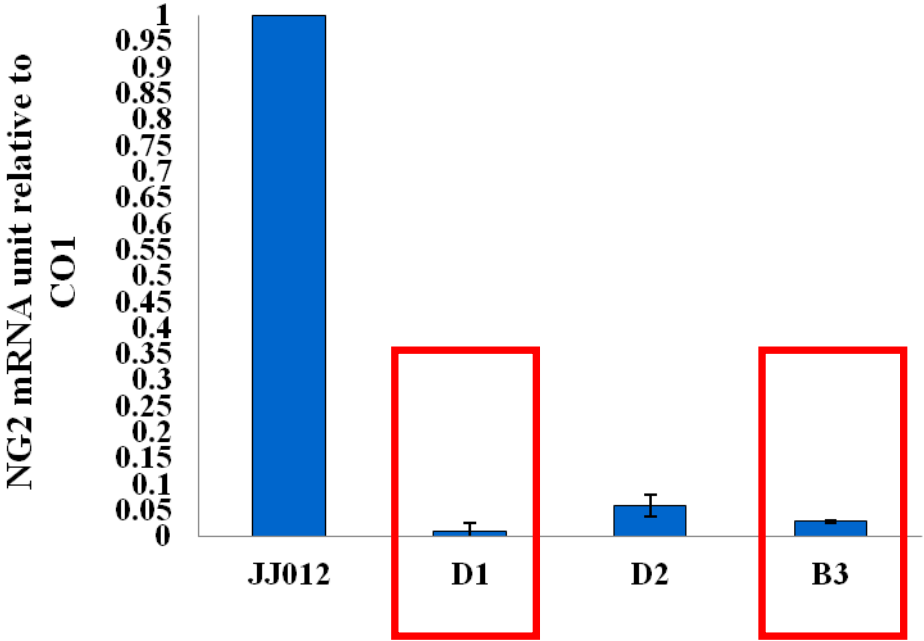


Figure 4.2: qPCR results for NG2/CSPG4 mRNA expression in JJ012 knocked-down cells (pooled). Cells were transfected with 5 different constructs (A, B, C, D and E) targeting NG2/CSPG4 and grown in culture. RNA was extracted and qPCR was carried out using NG2 primer. CO1 was used as a reference gene. NG2 mRNA unit shown in figure represents the relative expression of NG2/CSPG4 in knocked down cells relative to NG2+VE cells (JJ012). Error bars shown represent the SEM of triplicate wells for each cell type shown. The gene expression% maximum was shown in the table to the right side. **B and D constructs have the least NG2 expression among all constructs used.**

(a)



Gene expression %Max	
JJ012	100
D1	1.1
D2	5.9
B3	2.9

(b)

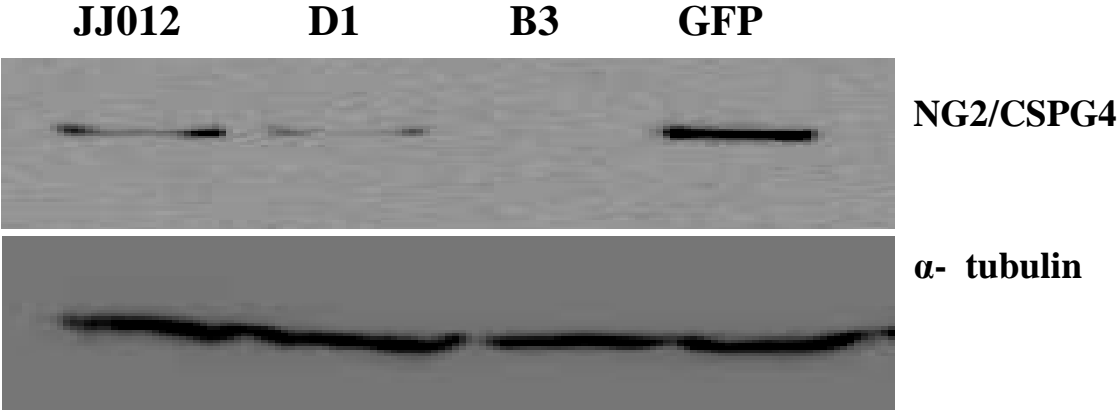


Figure 4.3: qPCR (a) and western blotting (b) results for NG2/CSPG4 mRNA expression in JJ012 knocked-down cells (2 and 1 clones from D and B constructs respectively). In image RNA was extracted, c DNA produced and qPCR was carried out using NG2 primer. CO1 was used as a reference gene. NG2 mRNA unit shown in figure represents the relative expression of NG2/CSPG4 in knocked down cells relative to NG2+VE cells (JJ012). Error bars shown represent the SEM of triplicate wells for each cell type shown. The gene expression % maximum was shown in the table on the right. Image b represents western blotting results for NG2/CSPG4 in B3, D1 and GFP cells. JJ012 was used as a positive control for western blotting. α -tubulin was used as internal control.

4.4 Assessment of NG2/CSPG4 knockdown by flow cytometry

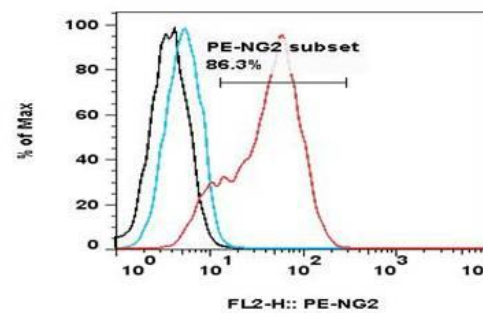
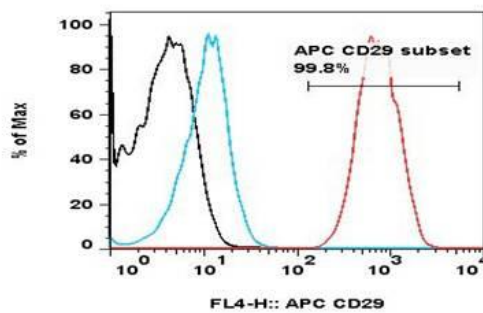
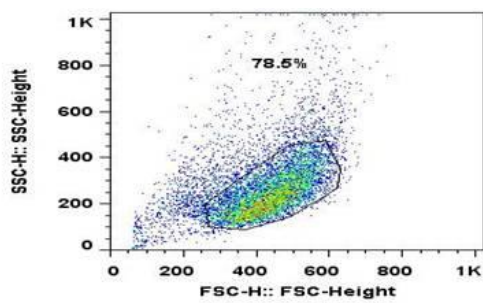
It was necessary to assess whether there was a surface protein expression of NG2/CSPG4 in D1 and B3 cells NG2 knocked down cells. Flow cytometry was carried out on JJ012 NG2+ve, D1 and B3 cells. Beta -1 integrin was used as a positive control. Cells were incubated with either NG2 antibody (LHM-2) or beta-1 integrin for 30 minutes before the analysis using the FACS Calibre machine (BD). Statistical analysis was carried out using One-way ANOVA.

Results showed that there was more than 99% expression of beta-1- integrin in all cells used. An average of three different independent experiments showed that the percentage of positive cells expressing NG2/CSPG4 on their surface was 85.86% for JJ012 NG2+VE, followed by D1(63.58%) and B3 (52.86%) (See figure 4.4 for representative image and figure 4.5 for the summary of the flow cytometry results). There was an extremely statistically significant difference (P value =0.0002) in the % of positive cells between JJ012 NG2+VE and B3 cells.

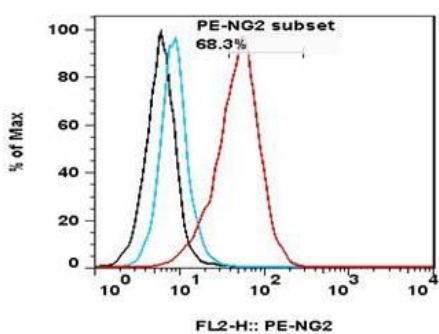
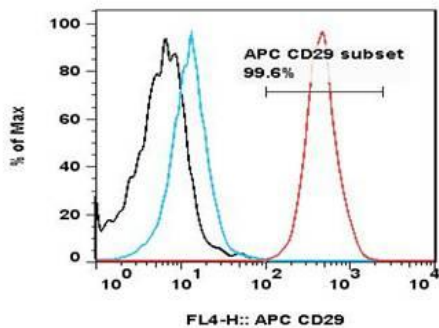
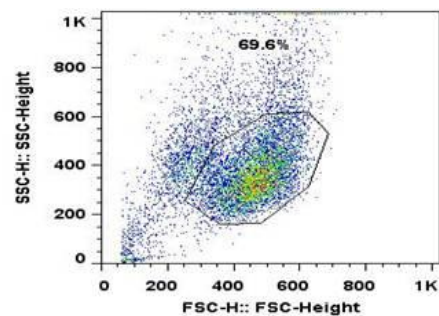
Analysis of the mean fluorescent intensity (MFI) from the same flow cytometry experiments showed that the MFI results for JJ012 NG2 +ve cells was 40.17, followed by 38.69 and 22.83 for D1 and B3 cells respectively. There was a very statistically significant difference (P value=0.005) in the MFI results between JJ012 NG2+ve cells and B3 cells.

It was necessary to investigate why B3 cells still express NG2 on their cell surfaces, while there was no detectable band of NG2 observed by western blotting. Therefore, flow cytometry was performed using a second antibody against NG2 (clone 9.2.27) (AB2). Results

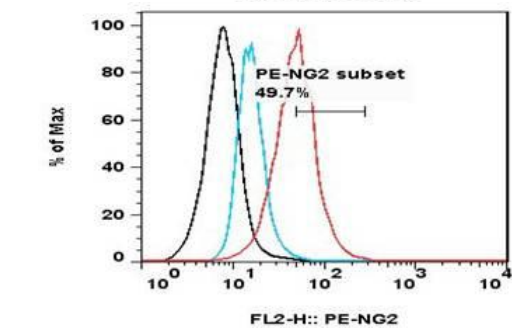
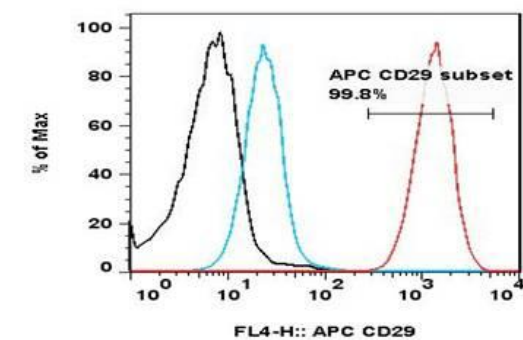
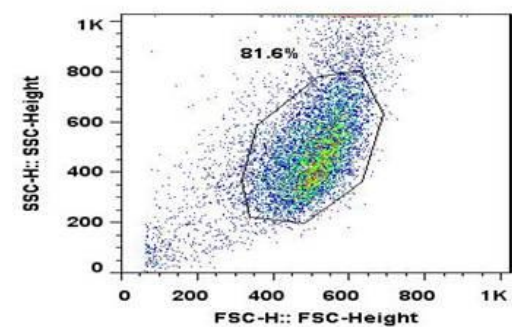
showed that there was no NG2/CSPG4 surface protein expression in B3 cells although JJ012 NG2+ve cells still maintained a high percentage of surface expression of NG2 (93.05%). Analysis of the MFI for the same samples showed that the MFI was 76.8 for JJ012, but nothing was observed in B3 cells.



JJ012



D1



B3

Figure 4.4: Representative images (n=3) of flow cytometry results (histogram analysis) for NG2/CSPG4 expression in JJ012 cells, D1 and B3 cells. CD29 was used as a positive control. Isotype antibody controls for NG2 (PE-A or FL2-H) and CD29 (APC-A or FL4-H) were used. Samples were analysed using FACS Calibre (BD). No antibody was added in unstained sample. In each image three coloured graphs have been shown: red= anti-NG2 or anti-CD29 antibody, Black= unstained and blue represents the isotype control. Percentages of positive cells for both NG2 and CD29 were shown in images presented.

Cell line	%positive cells
JJ012	85.86
D1	63.58
B3	52.86

(a)

Cell line	MFI (Isotype control)	MFI (NG2)	MFI (NG2)- MFI (Isotype control)
JJ012	5.66	40.17	34.51
D1	9.36	38.69	29.33
B3	8.65	22.83	14.18

(b)

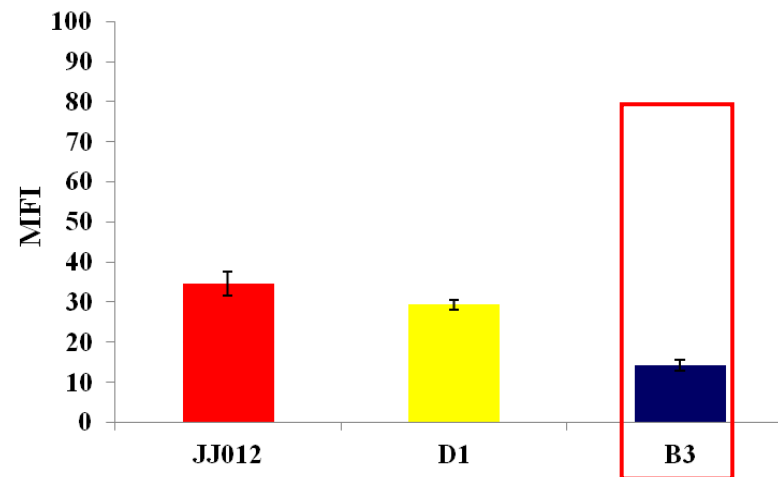
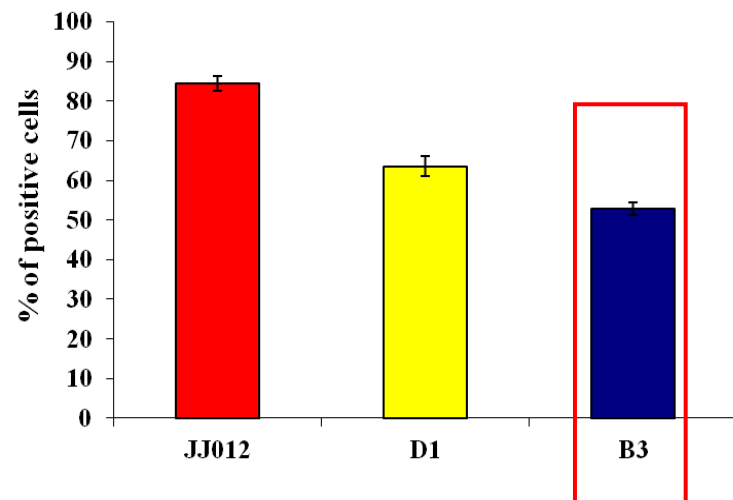


Figure 4.5: Summary of the flow cytometry results(n=3) for NG2/CSPG4 surface expression in JJ012 knocked-down cells(D1 and B3 cells) and JJ012 NG2+ve cells. The tables for the average of the % of positive cells and the mean fluorescent intensity (MFI) were illustrated in the table included (a) and (b). Results were shown as graphs for both % positive cells and MF1. There was an extremely and very statistically significant difference between JJ012 and B3 cells for both % positive cells and the MFI respectively.

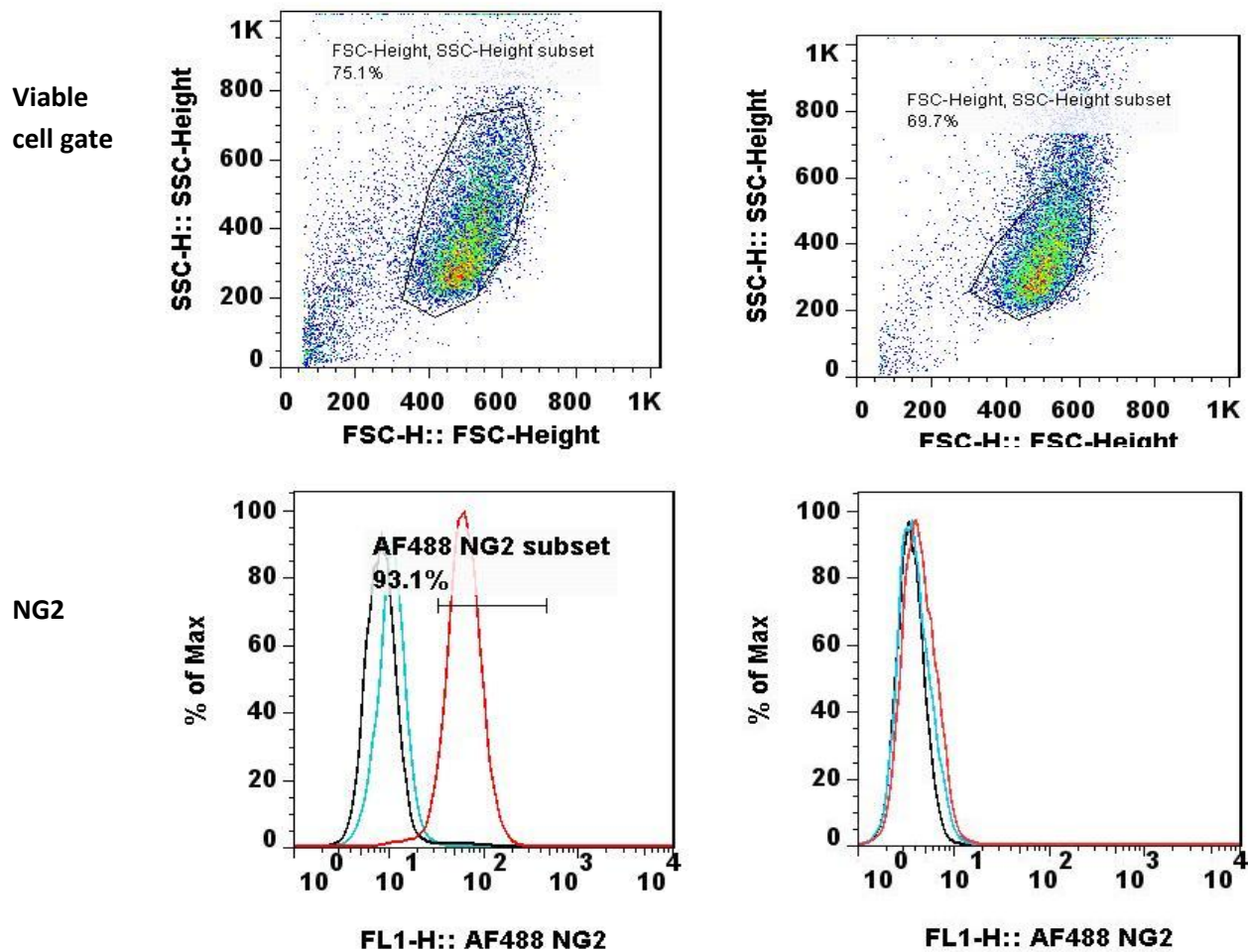


Figure 4.6: Representative image(n=3) of flow cytometry results for NG2/CSPG4 surface expression in JJ012 NG2+ve and B3 cells using 9.2.27 NG2 antibody as a primary antibody and Alexa flour 488 as a secondary antibody. Samples were analysed using FACS Calibre (BD). No antibody was added in unstained sample. In each image three coloured graphs have been shown: red= anti-NG2, Black= unstained and blue represents the isotype control. Percentages of positive cells for NG2 were shown in images presented.

Cell line	%positive cells
JJ012	93.05
B3	No NG2 expression

Cell line	MFI (Isotype control)	MFI (NG2)	MFI (NG2) - MFI (Isotype control)
JJ012	11.4	76.8	65.4
B3	10.7	9.5	-0.2

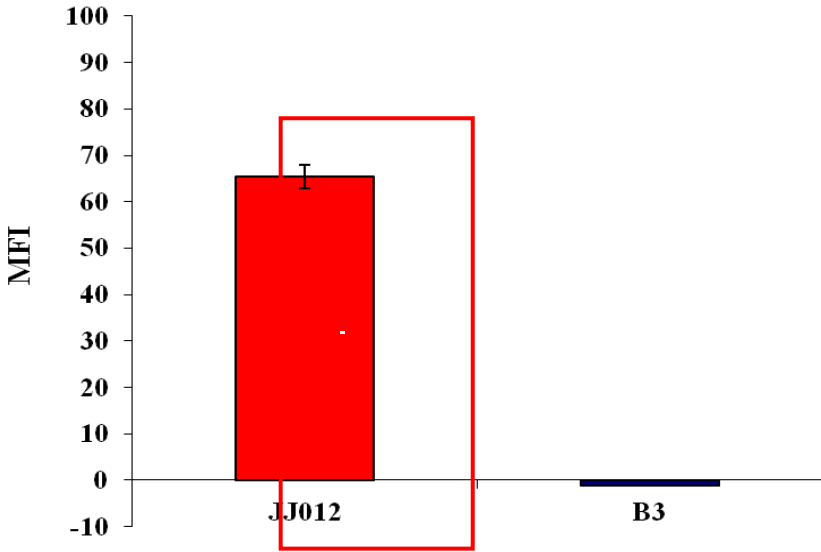
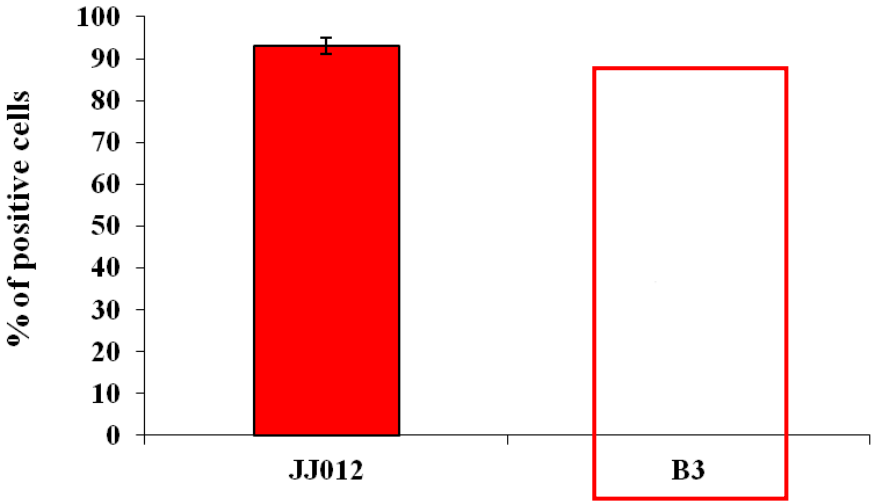


Figure 4.7: Summary of the flow cytometry results(n=3) for NG2/CSPG4 surface expression in JJ012 knocked-down cells(D1 and B3 cells) and JJ012 NG2+ve cells. The tables for the average of the % of positive cells and the mean fluorescent intensity (MFI) were illustrated in the table included (a) and (b). Results were shown as graphs for both % positive cells and MFI. There was a significant difference between JJ012 and B3 cells for both % positive cells and the MFI.

4.5 Assessment of the binding sites for the two NG2/CSPG4 antibodies used to assess the NG2 knockdown in JJ012

Different results of flow cytometry experiment for NG2/ CSPG4 expression were found. This happened by using different antibodies. Therefore, it was then necessary to determine whether the two NG2 antibodies: first (LHM-2) (labelled as AB1) and second (clone 9.2.27) (labelled as AB2) bind to the same or different epitopes on NG2.

Flow cytometry was carried out on JJ012 NG2+ve cells to demonstrate whether the binding epitopes for both NG2 antibodies are similar or different. Unstained (no antibody added) and isotype controls specific for each NG2 antibodies were used in this experiment. JJ012 cells were incubated with the AB2 (primary antibody added only without the addition of the secondary antibody (Alexa fluor-488) as used before) for 30 minutes followed by the addition of the AB1 (LHM-2, PE-Conjugated) for a further 30 minutes before analysing the samples on the FACS Calibre machine. Results are shown in figure 4.8

The data indicated, as expected, that there was still a high percentage (95.5%) of NG2 expression positive cells in JJ012 where both anti-NG2 antibodies were used. This was similar to the percentage of positive cells observed in JJ012 sample after being incubated with AB1 only (figure 4.6). This indicates that both antibodies bind to different epitopes on NG2/CSPG4. The addition of one NG2/CSPG4 antibody to the same sample sequentially did not inhibit the binding with their binding sites (i.e. there was no competitive inhibition of both NG2 antibodies).

Although the amino acid sequence of the epitope is separated in the primary structure, they are closely located in the three dimensional folding of the protein, Therefore, to further support this study's flow cytometry data, western blotting was carried out under reducing and non- reducing conditions, using the two NG2 antibodies used by flow cytometry.

Western blotting under reducing conditions for both NG2 antibodies showed that there was only one detectable band for NG2 in samples used (see figure 4.8). However, by using the LHM-2 NG2 antibody in western blotting under non reducing conditions, there was an additional NG2 band observed around 55kD together with the 270kD band. This was different from the results demonstrated by using (9.2.27) NG2 antibody, where there was only one single distinct band for NG2 detected at 270kD.

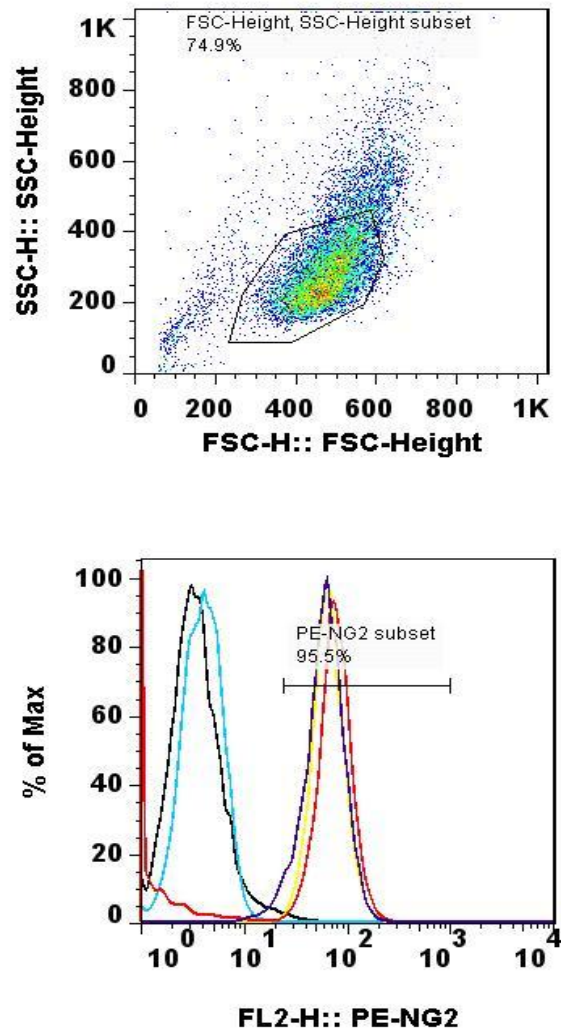


Figure 4.8: Flow cytometry results for NG2/CSPG4 surface expression in JJ012 (n=1) to demonstrate the binding sites for both NG2 antibodies used in western blotting and flow cytometry to assess NG2/CSPG4 knockdown. Samples were analysed using FACS Calibre (BD). No antibody was added in unstained sample. Results indicated that both antibodies bind to different parts of NG2. The colours shown in the image are illustrated in the box below the graph.

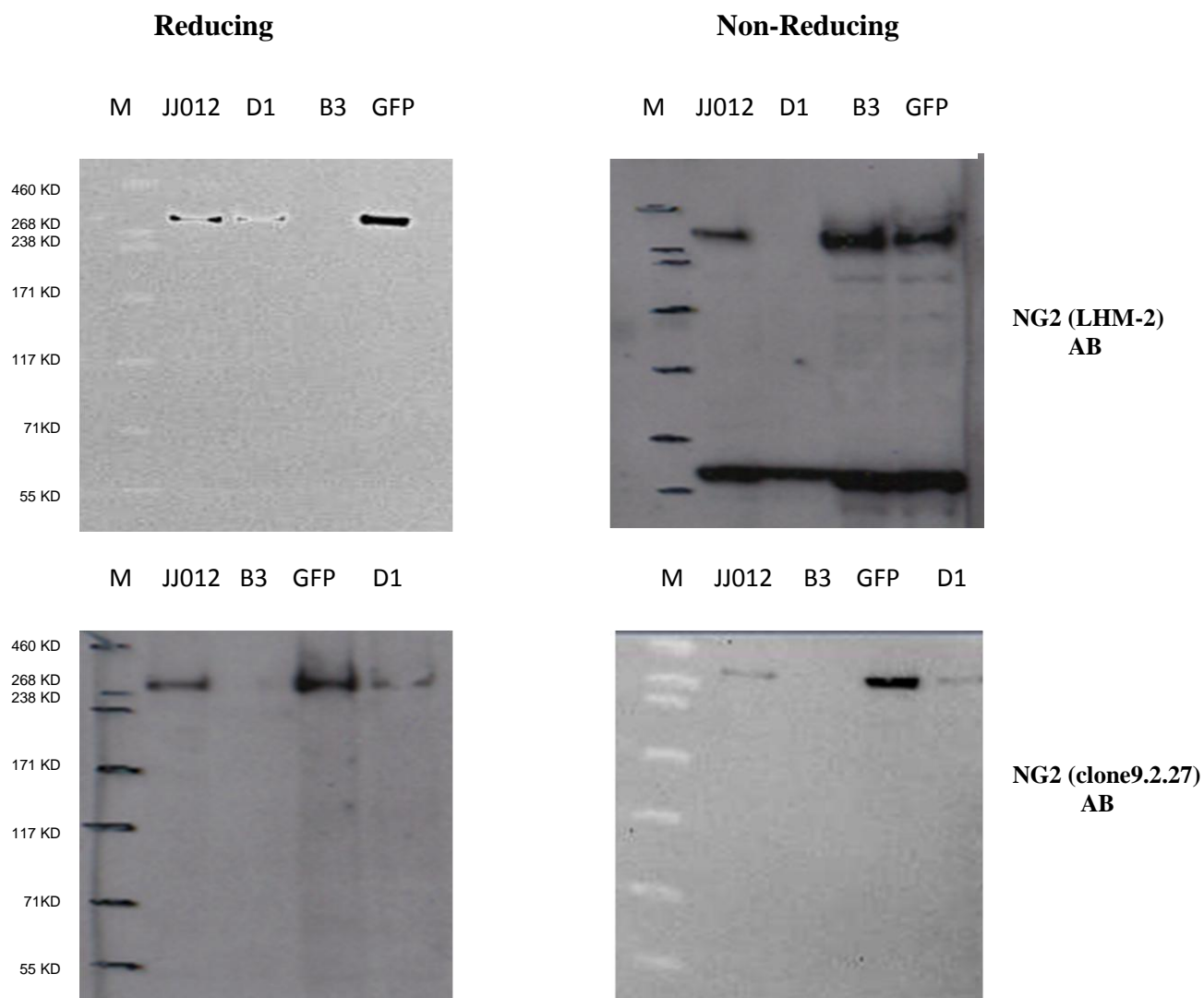


Figure 4.9: Western blotting results for NG2/CSPG4 protein expression in JJ012 and B3 cells under reducing and non-reducing conditions using the two different antibodies against NG2/CSPG4 (LHM-2 and clone 9.2.27 NG2 antibodies). Both antibodies showed one detectable band of NG2 at 270kD under reducing conditions. However, using (LHM-2) under non- reducing condition in western blotting, another NG2 band, around 55kD, was detected together with the 270kD band. Still only one distinct NG2 band was detected in western blotting under non reducing condition using clone (9.2.27) NG2 antibody.

4.6 Summary

NG2/CSPG4 gene knockdown was achieved in JJ012 chondrosarcoma cell line. The lowest expression was detected in B and D constructs, with 12 and 10 % of maximum gene expression respectively. Five colonies were then selected from the B and D construct bearing cell lines. NG2/CSPG4 gene expression was 2.9 and 1.1 % of maximum for cell lines B3 and D1 respectively. NG2/CSPG4 protein expression was undetectable in B3, while D1 cells showed a 270kD band consistent with expression of the core protein. The percentage of NG2 positive cells and mean fluorescent intensity were lowest for B3 cells in comparison D1, in comparison to JJ012 NG2 +ve cells.

4.7 Discussion

In this study, NG2/CSPG4 gene knockdown was achieved in JJ012 chondrosarcoma cell line. A B3 cell is the best model to study the effect of NG2 loss in human chondrocytes. Using NG2/CSPG4 (LHM-2) antibody in flow cytometry experiment, although there was an extremely and very statistically significant difference between JJ012 and B3 cells for both % positive cells and the MFI respectively, still there was a detectable level of NG2/CSPG4 expression in B3 cells. However, by using NG2 /CSPG4 antibody clone 9.2.27, there was no detectable level of NG2/CSPG4 expression in B3 cells.

There are few possible explanations why the first NG2 antibody still detected NG2/CSPG4 in B3 cells, while no detectable level of NG2 was observed in B3 cells by using the second NG2 antibody:

1. It is possible that the antibody detected break down of NG2 products.
2. Detected the same epitope which is the same for other proteins.

3. Recognised the same epitope which is available on different parts of NG2/CSPG4.

Therefore, to clarify and support these data further, it was then necessary to determine whether the two NG2 antibodies: first (LHM-2) and second (clone 9.2.27) bind to the same or different epitopes on NG2. Flow cytometry experiment to detect the binding sites of both NG2/CSPG4 antibodies and then western blotting experiment under reducing and non reducing conditions was performed. All these results proved that both NG2 antibodies bind to different sites of NG2 and further supported that B3 cells is the best model for NG2 knockdown.

CHAPTER FIVE

Results

5.0 The functional roles of NG2/CSPG4 in human chondrocytes

5.1 NG2 and ECM

5.1.1 The role of NG2/CSPG4 in adhesion

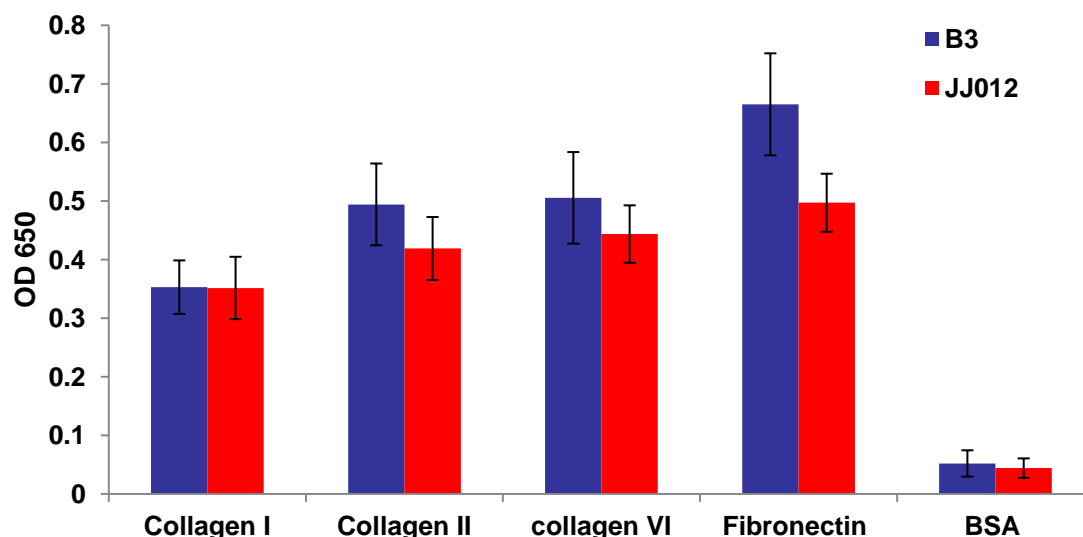
The role of NG2/CSPG4 in the adhesion process in human chondrocytes was assessed using methylene blue adhesion assay for JJ012 (NG2 +ve) and B3 (NG2-ve) cells. The role of integrins was assessed by using functional blocking antibodies: 1) $\beta 1$ integrin blocking antibody (Millipore, UK). 2) $\alpha V\beta 3$ integrin blocking antibody (Millipore, UK). EGTA and EDTA were used to characterise the divalent cation dependent and independent roles of NG2 in the adhesion process.

Human chondrosarcoma cell adhesion to ECM molecules

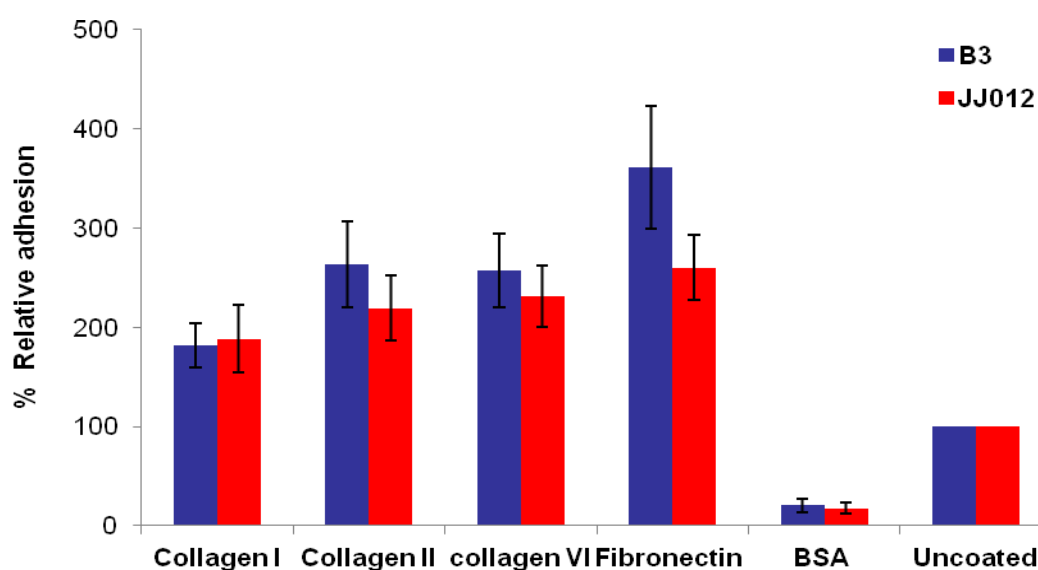
The selection of the dye, best seeding density and the optimised concentration of the BSA blocking agent were all performed before setting up the assay (data shown in the Materials and Method chapter). A standard curve was set up using different seeding densities. JJ012(NG2+ve) and B3(NG2-ve) cells were grown on different extracellular matrix molecules (ECM) in 96 well plates and allowed to adhere for an hour at 37°C before fixation with 4% PFD and staining with methylene blue dye. Extracellular matrix molecules used were: collagen I, II, VI and fibronectin. Each experiment was performed in triplicate and n=11 samples were done using cells grown at different passage numbers.

Results showed both JJ012 (NG2 +ve) and B3 (NG2-ve) cells adhere to collagen I, II, VI and FN coated wells. Nonspecific binding to BSA was very low in both cells. Statistical analysis was performed using Prism 3 software. Using Unpaired t-test, there was no significant difference in the adhesion percentages between JJ012 (NG2 +ve) and B3 (NG2-ve) cells to different ECM molecules used (Figure5.1).

In addition, One-way ANOVA test was used to compare the adhesion percentages to different ECM molecules in the same cell type, where collagen I was considered as a control. There was no significant difference in the adhesion ability of JJ012 (NG2+ve) to different ECM molecules used. However, in B3 (NG2-ve) cells, there was a statistically significant difference in the adhesion percentage between collagen I and FN only with a P value=0.0479.



Graph (A)



Graph (B)

Figure 5.1: No difference in the cell adhesion process between JJ012 (NG2+VE) and B3 (NG2-ve) cells. The graphs above illustrate the absorbance readings (graph A) and percentages of relative cell adhesion (graph B) for JJ012 (NG2+VE) and B3 (NG2-ve) cells. The values of cell adhesion presented in graph B for cells grown on collagen I, II and VI and FN were normalised to uncoated wells. Bars represent the values of eleven independent experiments (carried out at different passage numbers); each performed in triplicate \pm SEM. Non-specific binding to BSA was very low. Statistical analysis was carried out using unpaired t-test. No difference in the cell adhesion process was observed between JJ012 (NG2+VE) and B3 (NG2-ve) cells.

The effects of integrin blocking antibodies, EGTA and EDTA on the adhesion process

NG2/CSPG4 has been shown to interact with $\beta 1$ integrin and this interaction appears to influence different cellular processes, such as adhesion, motility, proliferation, as well as survival and responsiveness to chemotherapy (Midwood and Salter 1998, Fukishi et al 200, Makagiansar et al 2007, Chekenya et al 2008). Therefore, it was important to determine whether the adhesion process in JJ012 human chondrosarcoma cells occurs through NG2/CSPG4-integrin dependent activation, or through NG2 only dependent mechanism. JJ012(NG2+ve) and B3 (NG2-ve cells) were pre-treated with either $\beta 1$ integrin blocking antibody or $\alpha V\beta 3$ integrin blocking antibody for 30 minutes at 37°C before plating on different ECM molecules. Figure 5.2 and 5.3 show the results of this experiment. Statistical analysis using unpaired t-test was carried out in these experiments.

1. The effect of $\beta 1$ integrin blocking antibody

For statistical analysis, unpaired t-test was used. In the absence of $\beta 1$ integrin blocking antibody, JJ012 (NG2+ve) and B3 (NG2-ve) cells adhere to collagen I, II, VI and FN. $\beta 1$ integrin inhibited cell adhesion of both JJ012 (NG2+ve) and B3 (NG2-ve) cells to collagen I, however, there was no difference observed between both cell types. The adhesion of B3 (NG2-ve) cells appears to be increased on collagen II in the presence of $\beta 1$ integrin blocking antibody, while this antibody inhibited cell adhesion of JJ012(NG2+ve) to collagen II with an extremely, statistically significant difference observed between both cell types (P value= 0.0005).

However, pre-treatment of JJ012 (NG2+ve) and B3 (NG2-ve) cells with $\beta 1$ integrin reduced the cell adhesion ability of both JJ012 (NG2+ve) and B3 (NG2-ve) to collagen VI and fibronectin with a very statistically significant decrease in cell adhesion of JJ012NG2 +ve

cells in comparison to B3 (NG2-ve) cells (P value= 0.0031 and 0.0075 respectively). In addition, One-way ANOVA test was used to compare the adhesion percentages to different ECM molecules in the same cell type, where collagen I was considered as a control. There was an extremely, statistically significant difference in the adhesion ability of JJ012 (NG2+ve) to collagen I in comparison to FN, with a P value= 0.001. For B3 cells, there was a statistically extremely significant and significant difference in the adhesion percentage between collagen I and II and collagen I and VI, with P value= <0.0001 and 0.0194 respectively.

2. The effect of $\alpha V\beta 3$ integrin blocking antibody

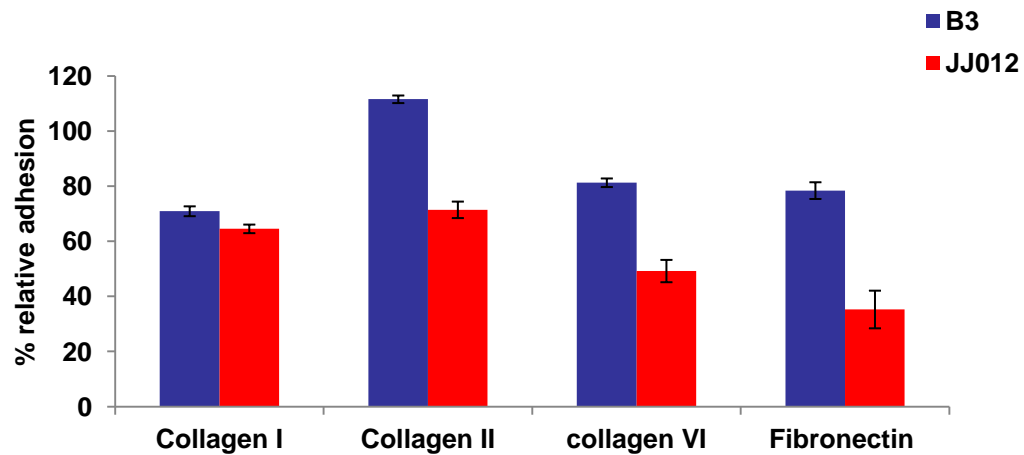
In the absence of $\alpha V\beta 3$ integrin blocking antibody, JJ012 (NG2+ve) and B3 (NG2-ve) cells adhere to collagen I, II, VI and FN. Pre-incubating the cells with $\alpha V\beta 3$ integrin blocking antibody (Figure 5.2) appears to inhibit cell adhesion of both JJ012 (NG2+ve) and B3 (NG2-ve) cells to different ECM used, with no statistically significant difference in the cell adhesion ability observed between both cell types or between different ECM molecules in the same cell type.

3. The effect of EDTA and EGTA

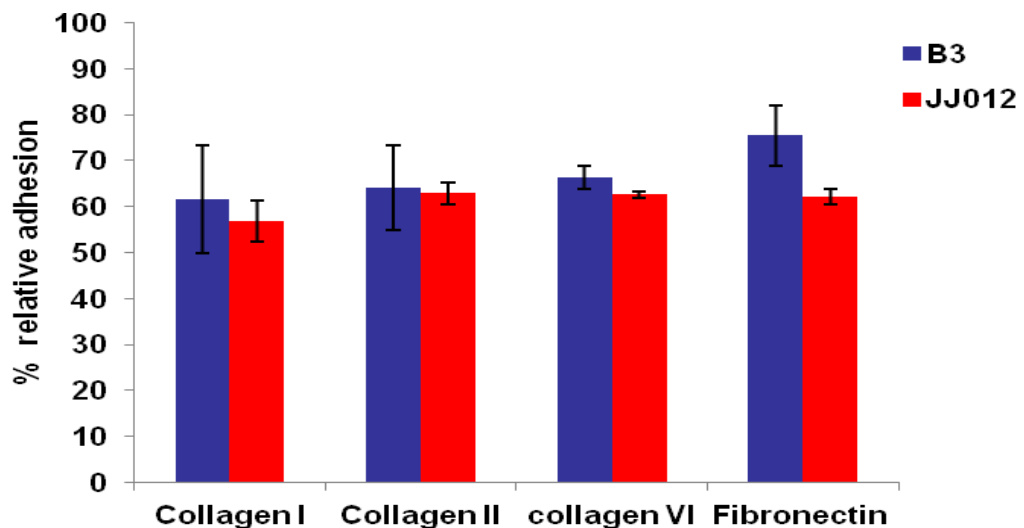
Cell adhesion molecules are classified as calcium dependent (such as integrins, cadherins and selectins) and calcium independent (such as members of the immunoglobulin super family). It is known that EDTA chelates divalent cations, such as magnesium, manganese and calcium, with low affinity for calcium compared to magnesium making it a poor candidate for studying the requirement of calcium in cellular activities. By comparison, EGTA is another chelator for divalent cations, which preferentially binds to calcium than other cations, with a significantly greater affinity for calcium than EDTA. Therefore, EGTA and EDTA can

be used to differentiate between calcium and magnesium dependent cellular activities. The effect of EDTA and EGTA on adhesion of JJ012 (NG2+ve) and B3 (NG2-ve) cells was carried out on fibronectin coated wells only. Cells were pre-treated with 5 and 10mM of EGTA or EDTA for 30 minutes at 37°C before plating on FN coated wells.

Figures (5.3 and 5.4) show the results of this experiment. Statistical analysis was carried out using unpaired t-test. In the absence of EDTA and EGTA, JJ012(NG2+ve) and B3 (NG2-ve) cells adhere to FN. Pre-treatment of both cell types with EGTA inhibited cell adhesion in a dose dependent manner, with no significant difference observed between both cell types. However, EDTA reduced adhesion of B3 (NG2-ve) cells but not JJ012 to fibronectin, with a more obvious difference observed at 10mM dose. 5mM EDTA significantly reduced adhesion of B3 cells but not JJ012 to fibronectin, with a P value= 0.009 (very significant). The difference was more obvious with 10mM concentration, with a P value=0.002 (very significant).



Graph (A)



Graph (B)

Figure 5.2: The involvement of $\beta 1$ (graph A) but not $\alpha V\beta 3$ (graph B) integrins in the adhesion process in human chondrosarcoma. The graphs above illustrate percentages of relative cell adhesion for JJ012 (NG2+VE) and B3 (NG2-ve) cells. The values of cell adhesion presented in both graphs for cells grown on collagen I, II and VI and FN were normalised to uncoated wells. Bars represent the values of three independent experiments (carried out at different passage numbers), each performed in triplicate \pm SEM. Statistical analysis was carried out using unpaired t-test. Non specific binding to BSA was very low. Statistical analysis was carried out using unpaired t-test and One-way ANOVA. There was a difference in the cell adhesion process between JJ012 (NG2+VE) and B3 (NG2-ve) cells, pre-treated with $\beta 1$ integrin blocking antibody. P values for the difference between B3 and JJ012 were: collagen II (0.0005, extremely significant), collagen VI (0.0031, very significant) and FN (0.0075, very significant).

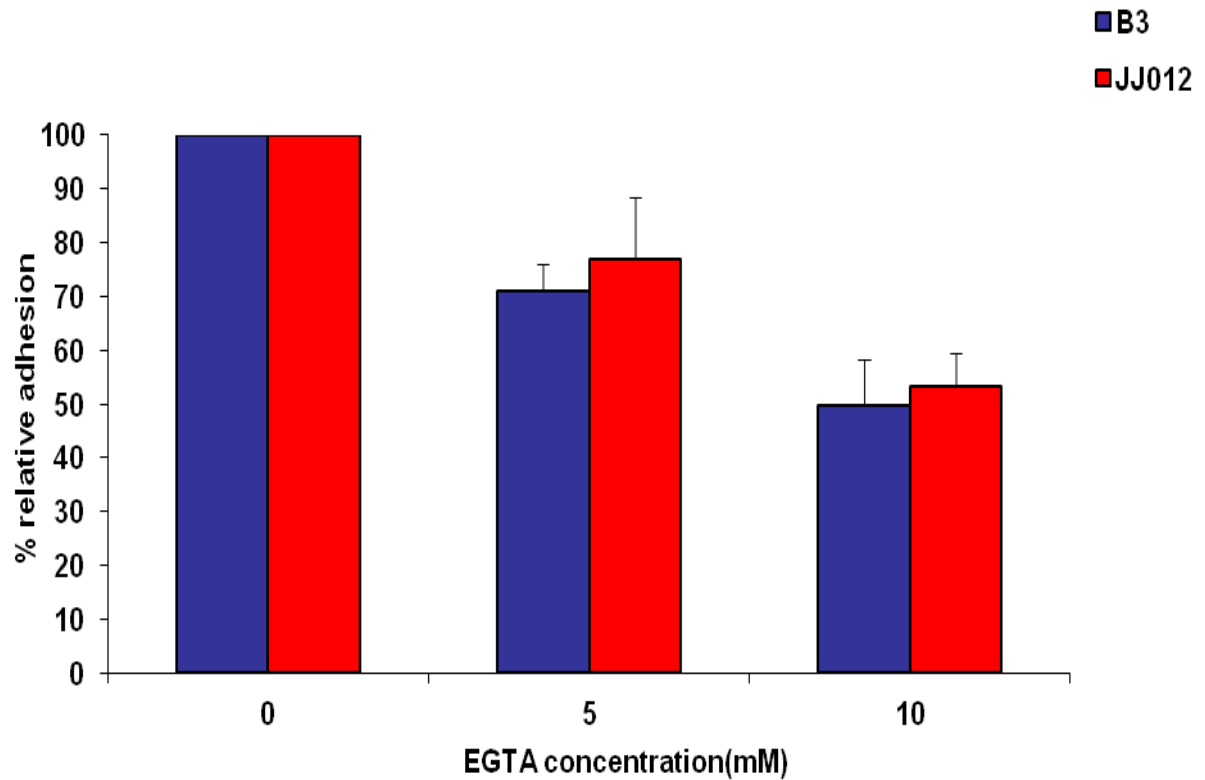


Figure 5.3: EGTA pre-treatment inhibited chondrosarcoma cell adhesion on FN coated well. There was an inhibition of cell adhesion in a dose dependent manner, with no difference observed between both cell types. JJ012 (NG2+ve) and B3 (NG2-ve) cells (n=3) were pre-treated with 5mM and 10mM EGTA concentrations and grown on fibronectin coated wells. Bars represent the values of three independent experiments (different passage numbers), each carried out in triplicate \pm SEM. Statistical analysis was carried out using unpaired t-test. Non specific binding to BSA was very low. EGTA inhibited cell adhesion in a dose dependent manner, with no difference observed between both cell types.

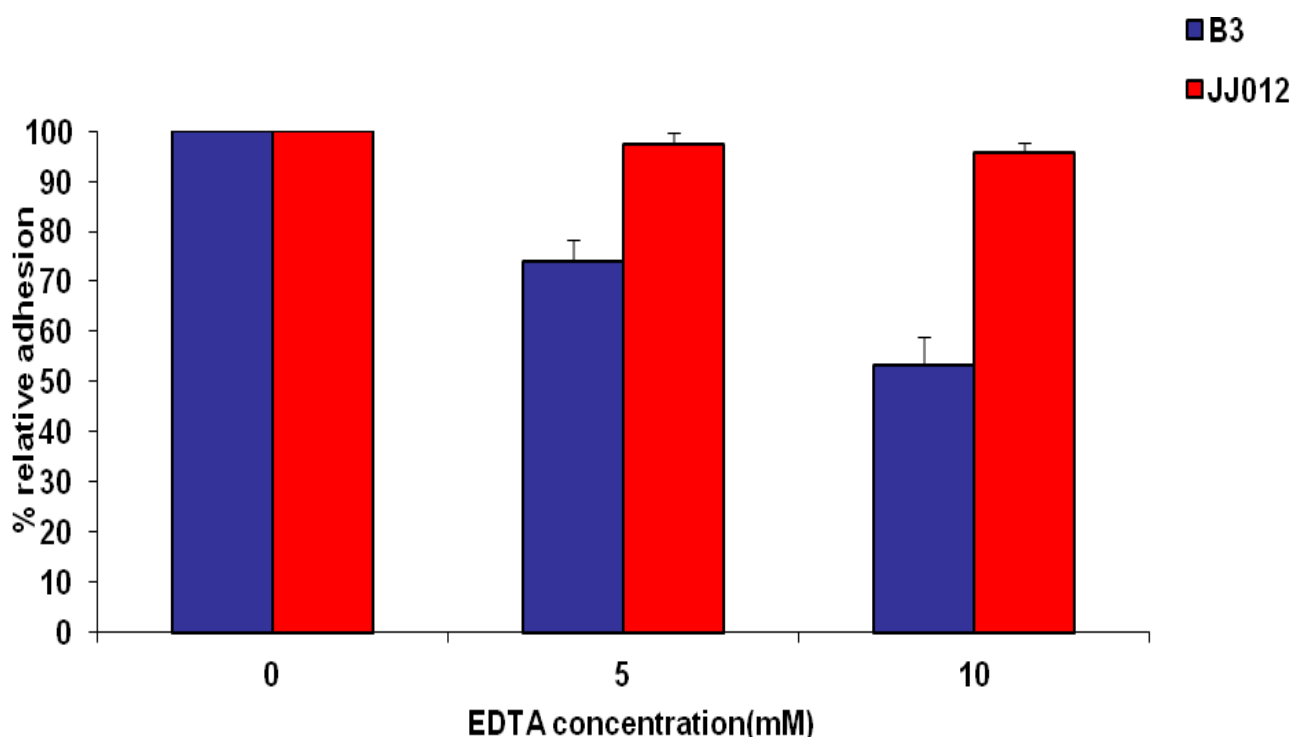


Figure 5.4: EDTA pre-treatment inhibited the cell adhesion of B3 (NG2-ve) cells, but not JJ012 (NG2+ve) on FN coated wells. The difference in cell adhesion between NG2+VE and NG2-VE was more obvious with 10mM concentration. JJ012 (NG2+ve) and B3 (NG2-ve) cells (n=3) were pre-treated with 5mM and 10mM EDTA concentrations and grown on fibronectin coated wells. Bars represent the values of three independent experiments (different passage numbers), each carried out in triplicate \pm SEM. Statistical analysis was carried out using unpaired t-test. Non specific binding to BSA was very low. 5mM EDTA significantly reduced adhesion of B3 cells but not JJ012 to fibronectin, with a P value= 0.009 (very significant). The difference was more obvious with 10mM concentration, with a P value=0.002 (very significant).

5.1.2 The role of NG2/CSPG4 in chondrocyte cell spreading

It has been shown that NG2/CSPG4 is able to promote cell spreading and motility on collagen VI. This further induces transmembrane signalling events that lead to actin cytoskeleton rearrangement and cytoplasmic signalling machinery (Stallcup review 2002).

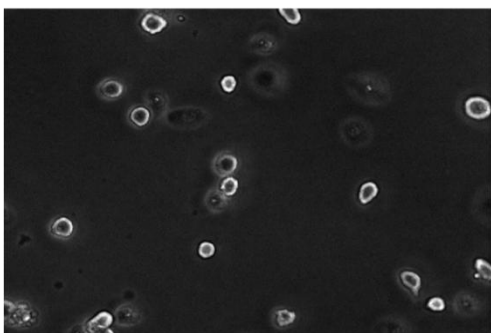
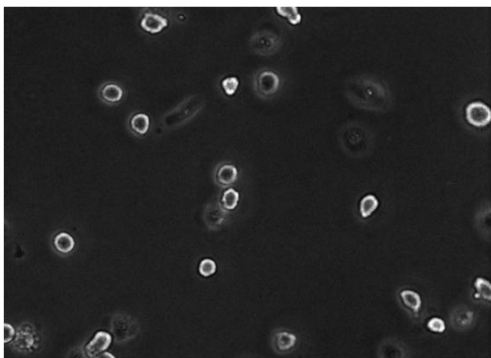
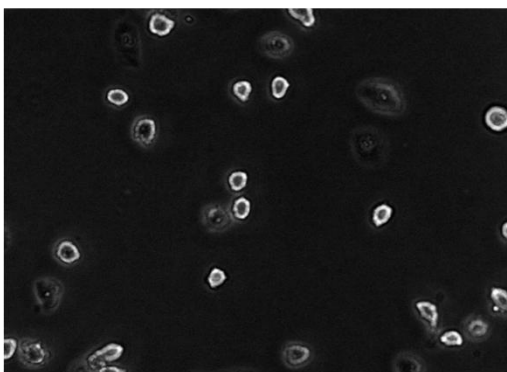
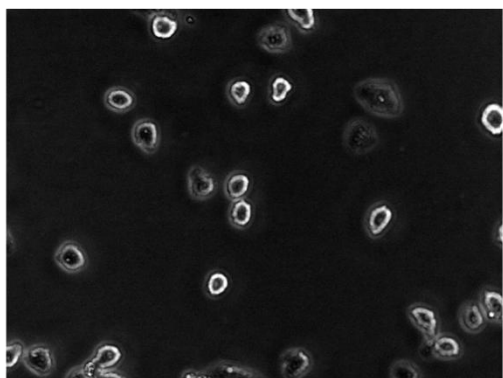
Cell imaging techniques using bright field imaging microscopy and a spreading assay were performed to characterise the spreading ability of JJ012 (NG2+ve) and B3 (NG2 -ve) cells on different ECM proteins: collagen VI, II and I and FN. For imaging, JJ012 (NG2+ve) and B3 (NG2 -ve) cells were seeded on FN coated wells in 12 well plates and images were taken after 15, 30, 45 and 60 minutes. For the cell spreading assay, JJ012 (NG2+ve), B3 (NG2 -ve) cells with the non-target cells were allowed to spread on different ECM proteins: Collagen I, II, VI and FN, fixed with 4% PFD and counted for cell spreading. Non-target cells (control) were used to exclude the off target effect of the NG2 knockdown process. Each spreading assay was done in triplicate and three independent experiments were performed using cells grown at different passage numbers. Cell spreading assessment was performed following the criteria of Martin J Humphries (Cell Adhesion Assays, chapter 14, link: <http://www.springerlink.com/content/w04q383563443208/#section=54595&page=2&locus=5>). Cells were regarded as spreading cells when the cell body is phase dark and the cytoplasm is visible around the entire circumference of the nucleus.

The preliminary data showed that B3 (NG2-ve) cells spread more than JJ012 (NG2+ve) on the ECM protein FN, with the most obvious difference observed after 60 minutes (figure 5.5). Subsequent studies using cell spreading assay (figure 5.6) further supported this data. Statistical analysis was performed using Prism 3 software. Using One-way ANOVA,

although both JJ012(NG2+ve) and B3 NG2 -ve cells spread on the different ECM proteins FN, collagen VI, II and I, B3(NG2-ve) cells spread more than JJ012(NG2+ve) on each of the different ECM proteins FN, Collagen VI, II and I. There was a statistically significant difference in the cell spreading ability of JJ012 (NG2+ve) in comparison to B3 (NG2-ve). There was no difference in the spreading ability of both JJ012 (NG2+ve) and control cells (non-target). The P values were: $P < 0.0001$ (extremely significant), 0.008 (very significant), 0.0008 (extremely significant) and 0.0003 (extremely significant) for collagen I, II, VI and FN respectively.

In addition, One-way ANOVA test was used to compare spreading ability to different ECM molecules in the same cell type, where collagen I was considered as a control. There was no significant difference in the adhesion ability of B3 (NG2-ve) cells to different ECM molecules used. However, in JJ012 (NG2+ve) cells, there was a statistically very significant, significant and very significant difference in the adhesion percentage between collagen I and II, collagen I and VI and collagen I, with P value = < 0.0043 , 0.0142 and 0.0077 respectively.

JJ012



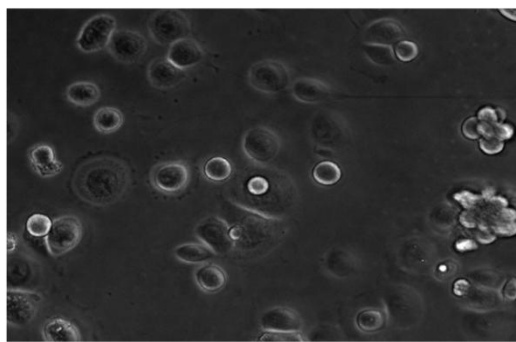
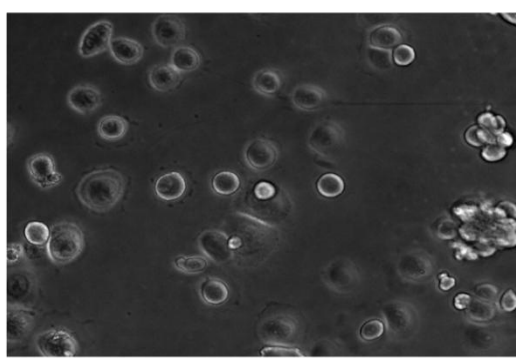
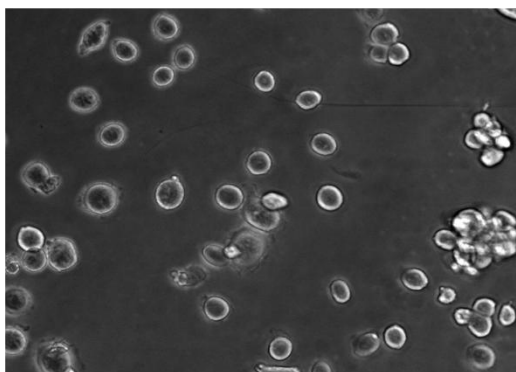
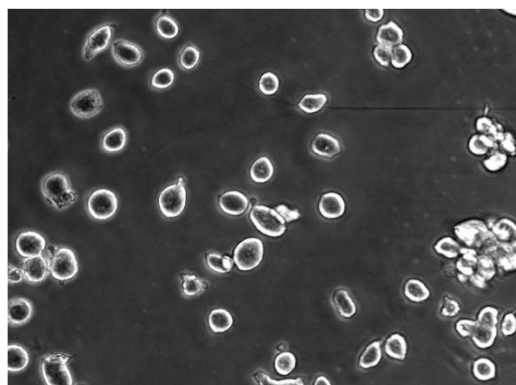
0 minutes

15 minutes

30 minutes

45 minutes

B3



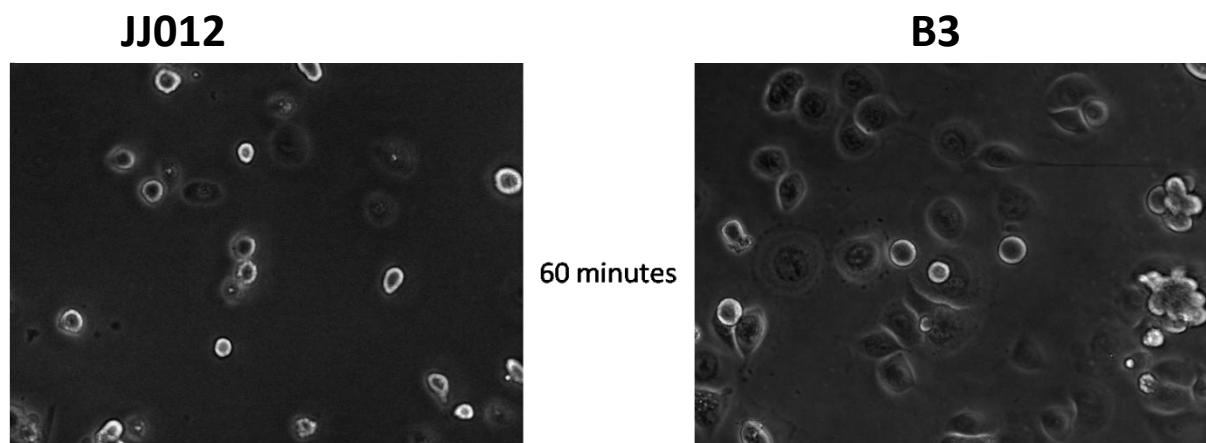


Figure 5.5: B3 (NG2-ve) cells spread more than JJ012 (NG2+ve) on ECM protein FN. Representative images for cell spreading of both JJ012 (NG2+ve) and B3 (NG2-ve) on fibronectin coated plates. The images were carried out over an hour at 15 minutes interval using the bright field microscope. Results indicated that B3 (NG2-ve) cells spread more than JJ012 (NG2+ve) on ECM protein FN.

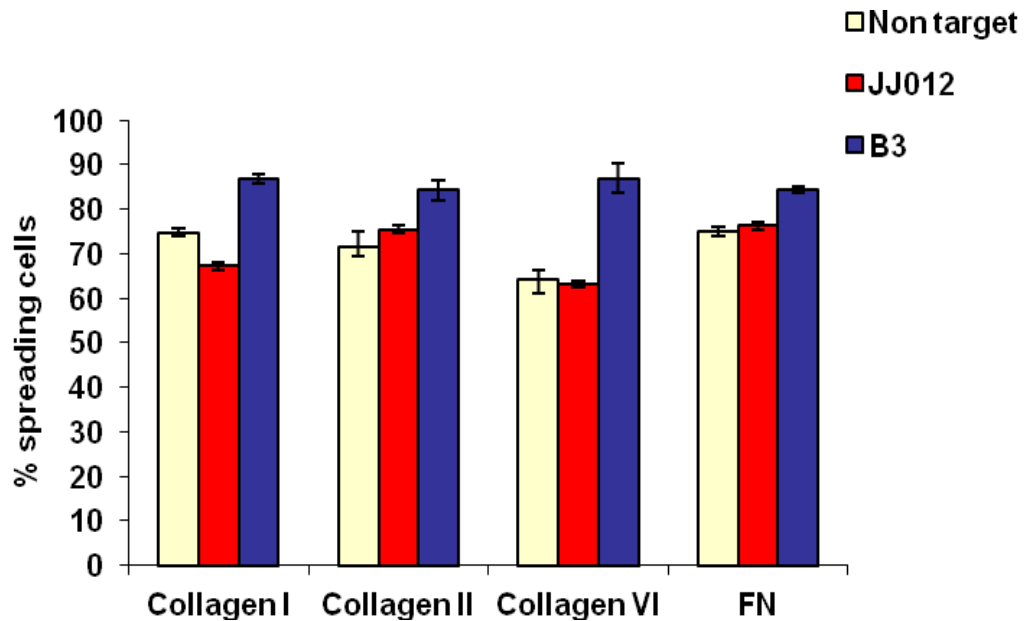


Figure 5.6: B3 (NG2-ve) cells spread more than JJ012 (NG2+ve) on different ECM proteins (collagen I, II and VI and FN) using cell spreading assay. The graph shows the percentages of cell spreading for JJ012 (NG2+VE), B3 (NG2-ve) and control cells (Non-target) cells (n=3). The percentages of cell spreading were normalised to the total number of cells. Bars represent the values of three independent experiments (where cells grown at different passage numbers), each carried out in triplicate \pm SEM. Statistical analysis was carried out using One-way ANOVA. B3 (NG2-ve) cells spread more than JJ012 (NG2+ve) on each of the different ECM proteins used. There was no difference in the spreading ability of both JJ012 (NG2+ve) and control cells (non-target). The P values were: $P < 0.0001$ (extremely significant), 0.008 (very significant), 0.0008 (extremely significant) and 0.0003 (extremely significant) for collagen I, II, VI and FN respectively.

5.1.3 The role of NG2/CSPG4 in chondrocyte cell migration

NG2/CSPG4 serves as a receptor or co-receptor for growth regulators; adhesion, migration, and invasion promoters; angiogenesis promoters; extracellular matrix glycoprotein and collagens; and enzymes that can cleave many of the receptors and matrix molecules. Our previous results showed roles for NG2/CSPG4 on cell adhesion and spreading. Therefore, we explored the effect of NG2/CSPG4 on migration of JJ012 (NG2+ve) and B3 (NG2-ve) cells using a wound healing technique.

JJ012 (NG2+ve), B3 (NG2 -ve) cells and non-target cells were grown in 12 well plates till they were confluent. Then cells were wounded by micropipette and images of the wounded area were taken immediately and after 10 hours of the scratch. Non-target cells (control) were used to exclude the off target effect of the NG2 knockdown process. Experiments were carried out in triplicate using cells grown at different passage numbers.

Results (figure 5.7) showed that B3 (NG2-ve) cells migrate less than JJ012 (NG2+ve) cells; with a very statistically significant difference observed in migration (P value=0.0013) (after 10 hours: the closed area was 81.4% for JJ012 (NG2+ve), 54.6% for B3 (NG2-ve and 82.2% for non-target cells.

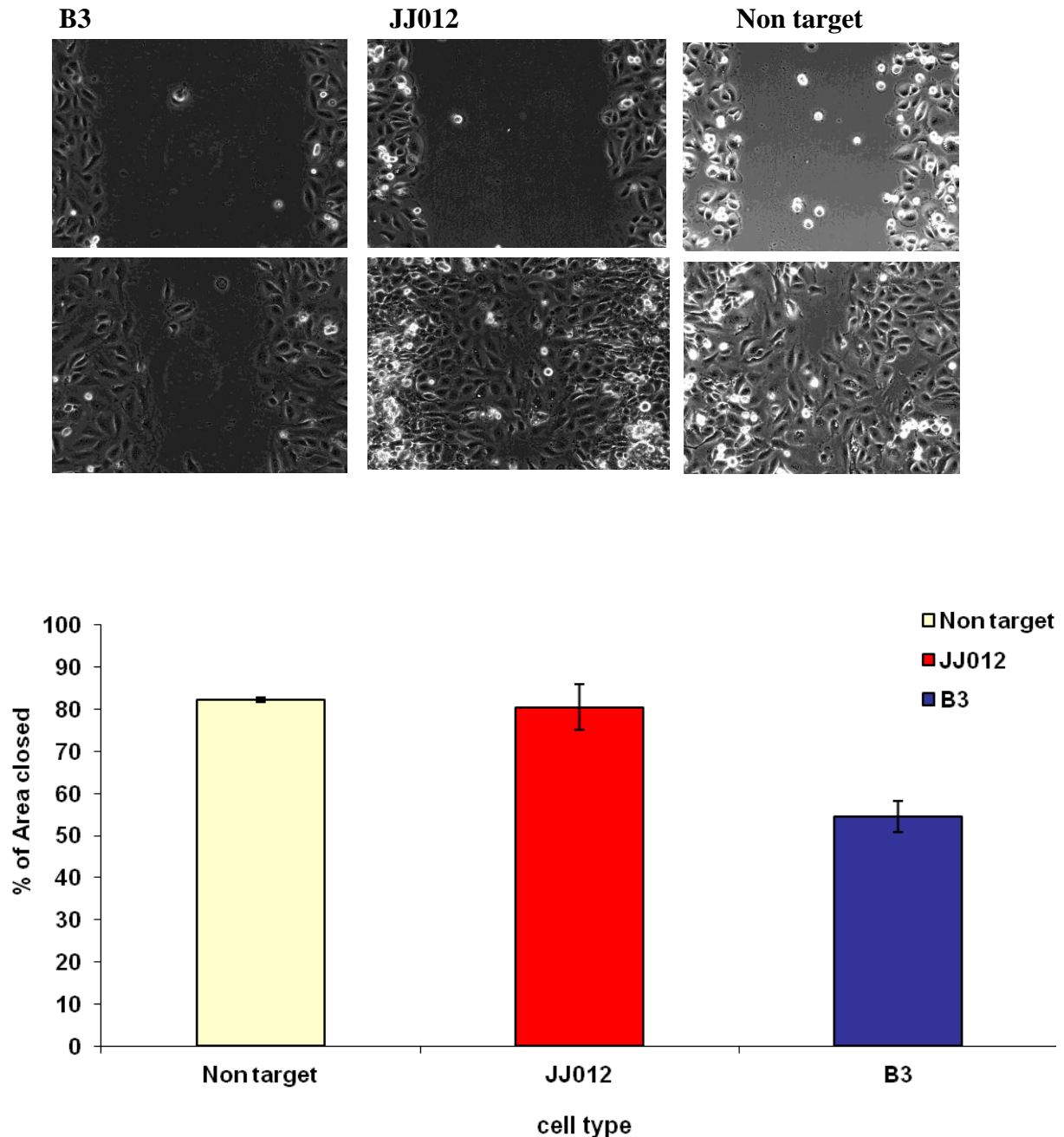


Figure 5.7: B3 (NG2-ve) cells migrate less than JJ012 (NG2+ve) using wound healing assay. Representative graph (n=3) illustrating the difference in cell migration between JJ012 (NG2+ve) and B3 (NG2-ve), using non-target cells as a control. The wound closure after 10 hours was assessed using T scratch software. The results were presented as a percentage of area closure after 10 hours normalised to immediately taken images after the scratch. The difference was very statistically significant (p value=0.0013).

5.1.4 The role of NG2/CSPG4 in chondrocyte cell invasion using an inverse invasion assay

JJ012(NG2+ve), B3(NG2 -ve) cells together with the non target cells were grown in inverted trans wells containing growth factor reduced matrigel for 5 days. Cells were then imaged using a confocal microscope. Non target cells (control) were used to exclude the off target effect of the NG2 knockdown process. Each experiment was carried out in triplicate using cells grown at different passage numbers. Limited number of samples was used due to technical problems and the shortage of time, standard deviation (STD) was presented in the graph below. For JJ012 (NG2+ve) and non target cells, N=2 samples were used, while n=3 samples were used for B3 (NG2-ve) cells.

Results are shown in figure (5.8). Results indicated that, although there were only n=2 samples for JJ012 cells, the data were consistent; and there appeared to be a difference between JJ012+ serum in comparison to JJ012+EGF. However, for n=3 samples of B3 cells, the variation was high and it was not possible to tell whether there was a difference between B3+serum and B3 +EGF. Similar results were obtained with non target cells.

To conclude and with the limited number of samples, the preliminary data showed that there was no difference in the cell invasion between B3 (NG2-ve) cells and JJ012 (NG2+ve) using matrigel invasion assay. The experiment should be repeated in more n of samples to support these results.

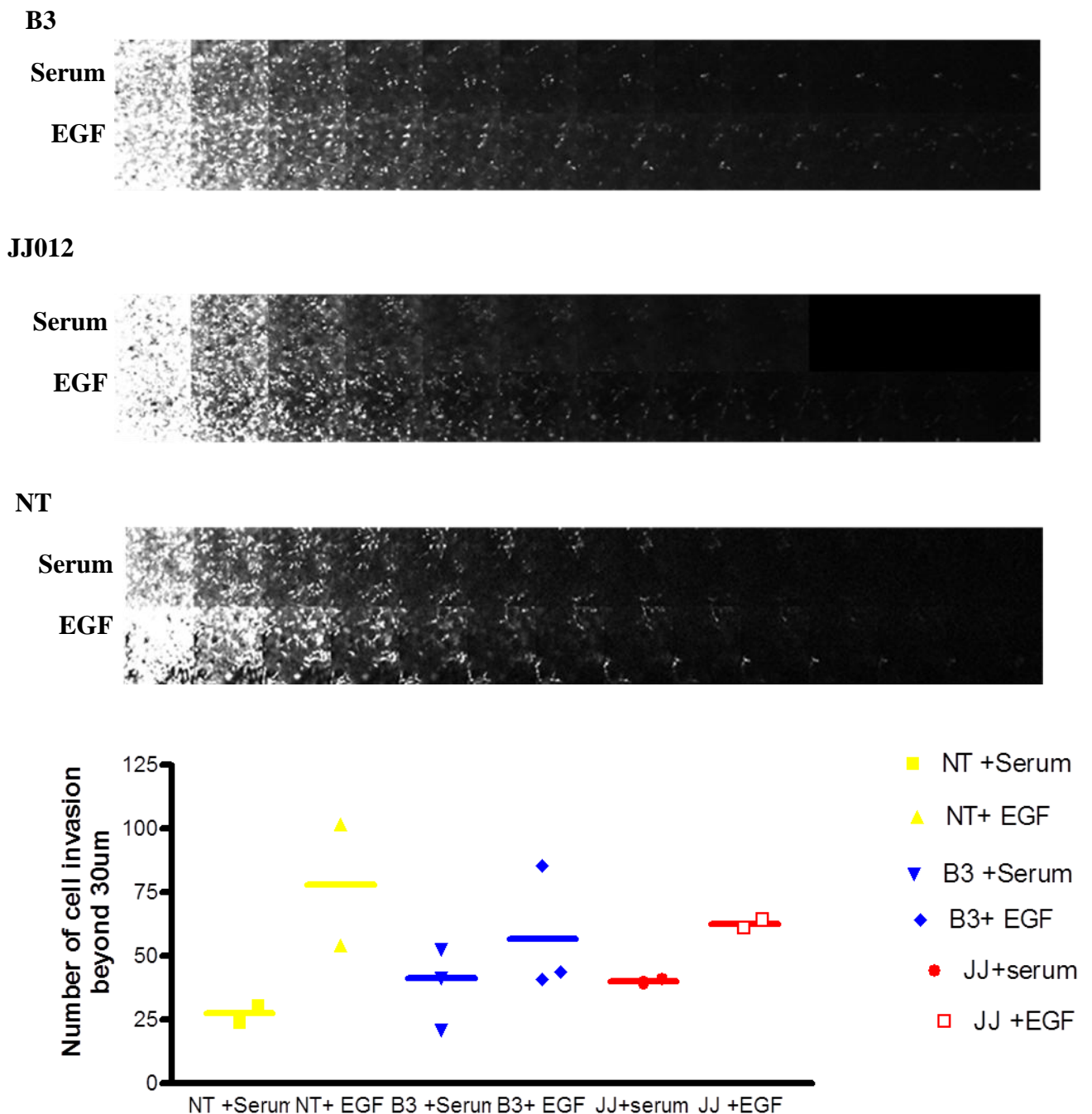


Figure 5.8: No difference in the cell invasion between B3 (NG2-ve) cells and JJ012 (NG2+ve) using matrigel invasion assay. Increased cell invasion (statistically significant difference) of stimulated JJ012 (NG2+ve) cell with EGF in comparison to un stimulated cells, while no difference in cell invasion was observed in B3+ serum and B3 +EGF. Representative graph (n=3) for B3 and n=2 for JJ012 and non target cells. Cells were grown and allowed to invade the matrigel for 5 days, then images were taken at 15µm thickness using confocal microscope. Quantification of cells invaded matrigel was carried out using Image J. The cells at level 0 thickness of matrigel was considered as 100 and the % of invaded cells in each section downwards was normalised to 0 level of matrigel. The bars shown represent the average of two or three independent experiments for cells grown at different passages, each carried out in triplicate.

5.1.5 The role of NG2/CSPG4 in chondrocyte cell viability, apoptosis and proliferation

Different studies demonstrated the involvement of NG2/CSPG4 cell proliferation in both normal and diseased conditions (Nishiyama et al 1996, Grako et al 1999, Stallcup review 2002 and Ozerdem and Stallcup 2004). This was supported by the fact that NG2/CSPG4 is capable of interacting with high affinity to the growth factors: FGF-2(bFGF) and PDGF-AA through its core protein part rather than the chondroitin sulphate chain (Goretzki et al 1999).

Therefore, it was necessary to investigate whether NG2 is involved in chondrocyte cell proliferation. To assess the role of NG2/CSPG4 in chondrocyte cell proliferation, JJ012 (NG2+ve), B3 (NG2 -ve) cells together with the non-target cells were grown in culture for 24 and 48 hours. Then, MTT assay or CyQUANT® Cell Proliferation assay were carried out. Statistical analysis was carried out using Unpaired t-test or One-way ANOVA.

MTT results showed that B3 (NG2-ve) cells proliferate less than JJ012 (NG2+ve), with an extremely statistically significant difference in the proliferation rate between JJ012 (NG2+ve) and B3 (NG2 -ve) cells at 48 hours. This is further supported by CyQUANT® Cell Proliferation assay results, which demonstrated the same cellular response. There was no difference in the proliferation rate between JJ012 (NG2+ve) cells and non-target cells using both assays. Figures 5.9 and 5.10 show the results of these experiments.

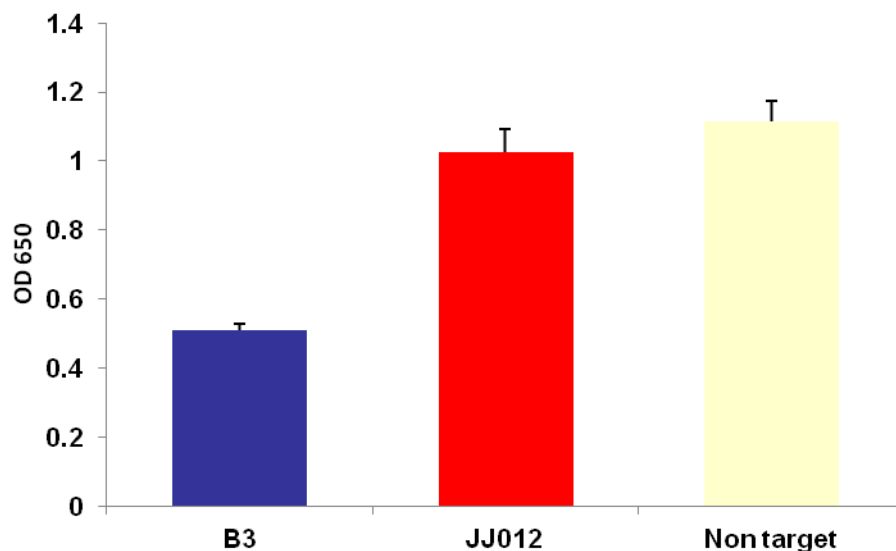


Figure 5.9: B3 (NG2-ve) cells proliferate less than JJ012 (NG2+ve) using MTT assay. MTT was used to demonstrate the difference in cell proliferation between B3 (NG2-ve) and JJ012 (NG2+ve) cells. There was an extremely statistically significant difference in cell proliferation between B3 (NG2-ve) and JJ012 (NG2+ve), with a p value= <0.001 .

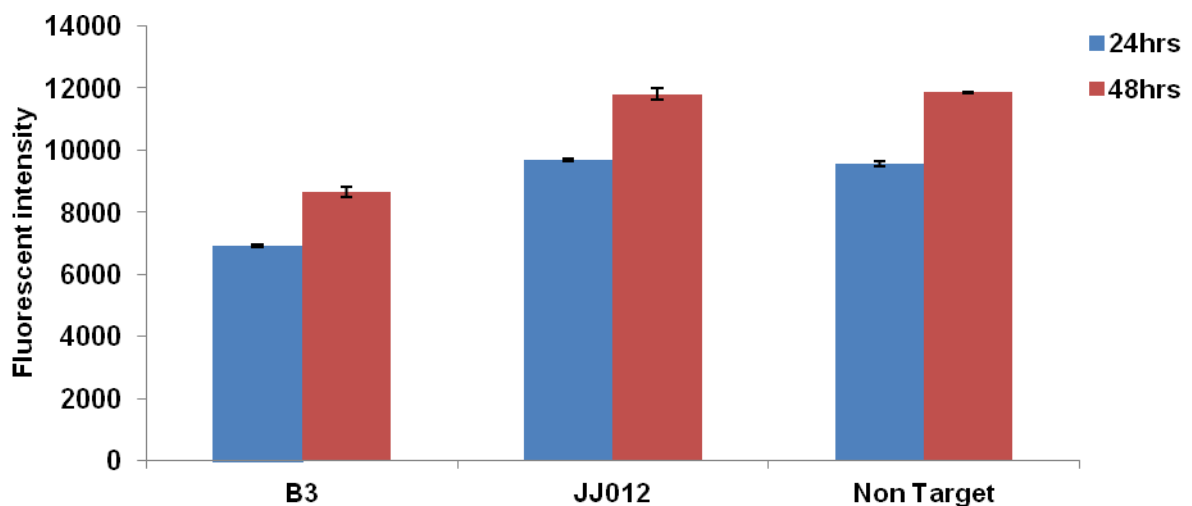


Figure 5.10: B3 (NG2-ve) cells proliferate less than JJ012 (NG2+ve) using CyQUANT® Cell Proliferation assay. CyQUANT® Cell Proliferation assay was carried out using five thousand cell seeding density at 24 and 48 hours. Bars represent the values of three independent experiments (where cells grown at different passage numbers), each carried out in triplicate \pm SEM. The proliferation rate was significantly reduced at 24 hours followed by 48 hours in B3 (NG2-ve) cells compared to JJ012 and non-target cells. There was an extremely statistically significant difference (p value <0.0001) between JJ012 (NG2+ve) and B3 (NG2-ve) cells.

The apoptotic death of articular chondrocytes has been implicated in the pathogenesis of osteoarthritis (Zamli and Sharif 2011) and the role of NG2/CSPG4 in the modulating apoptotic cell deaths started to be explored. NG2/CSPG4 can act as pro-apoptotic or anti-apoptotic receptors (Chekenya et al 2011, Chekenya et al 2008 and Joo et al 2007). However, it is not known yet whether NG2 is involved in the apoptotic mechanism in OA or chondrosarcoma.

To assess the role of NG2/CSPG4 in chondrocyte cell apoptosis, JJ012 (NG2+ve), B3 (NG2 -ve) cells together with the non-target cells were grown in culture for 24 and 48 hours. Then, flow cytometry using annexin V staining was carried. Statistical analysis was carried out using Unpaired t-test or One-way ANOVA

Results showed that there was no statistically significant difference in the apoptosis percentages between JJ012 (NG2+ve) and B3 (NG2 -ve) cells at both time points. There was also no difference in the apoptosis percentage between JJ012 (NG2+ve) cells and non-target cells. Figure5.11 shows the results of this experiment.

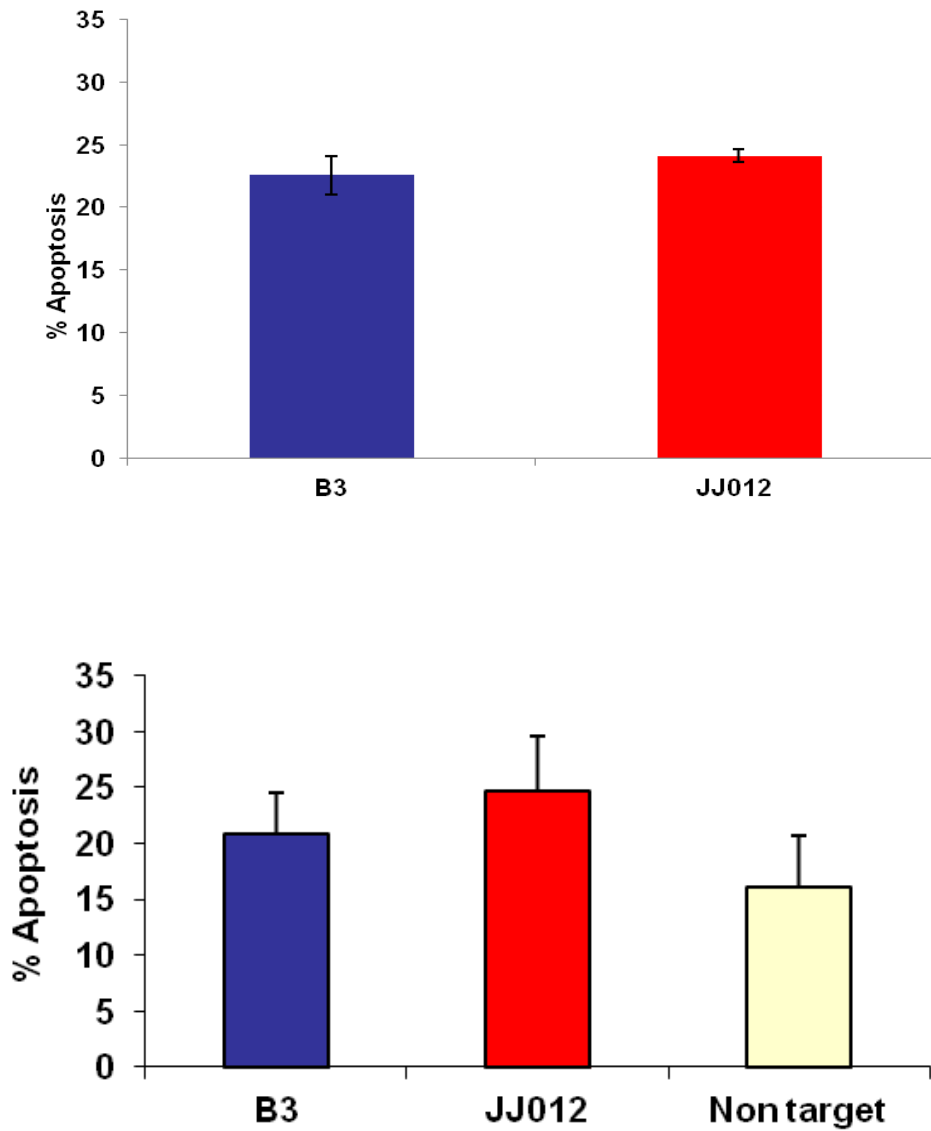


Figure 5.11: No difference in cell apoptosis between JJ012 (NG2+ve) cells and B3 (NG2-ve). Flow cytometry was used to assess the percentages of apoptosis (Annexin V +ve cells) in B3 (NG2-ve) and JJ012 (NG2+ve) cells at 24(n=4+/-SEM) and 48 hrs (n=10 +/-SEM for B3 and JJ012 and n=4 +/-SEM for non-target) respectively.

5.2 The role of NG2/CSPG4 in chemoresistance and apoptosis

5.2.1 Optimisation of the seeding density and time point for assessment of the chemotherapeutic agents on JJ012 (NG2+ve) cells

We hypothesised that NG2/CSPG4 may be involved in the chemoresistance in human chondrosarcoma. To test our hypothesis, first of all, it was necessary to select the best seeding density and the optimal time point to set out the dose response curve for the chemotherapeutic drugs to be used. JJ012 (NG2+ve) cells were plated in 96 well plates and incubated for 24, 48 and 72 hours. Cell growth was assessed by MTT assay. Three cell densities were used in this experiment (2.5 , 1.25 and $0.625 \times 10^4/\text{ml}$) with $100\mu\text{l}$ / well. Results (figure 5.12a, b and c) showed that 1.25×10^4 seeding density at 48 hours were the best seeding density and the time point to be used for testing the response of human chondrosarcoma cells to different doses of chemotherapeutic drugs to be used. We chose 1.25×10^4 seeding density as the other two seeding densities may show either small or saturated effect of drugs on cells.

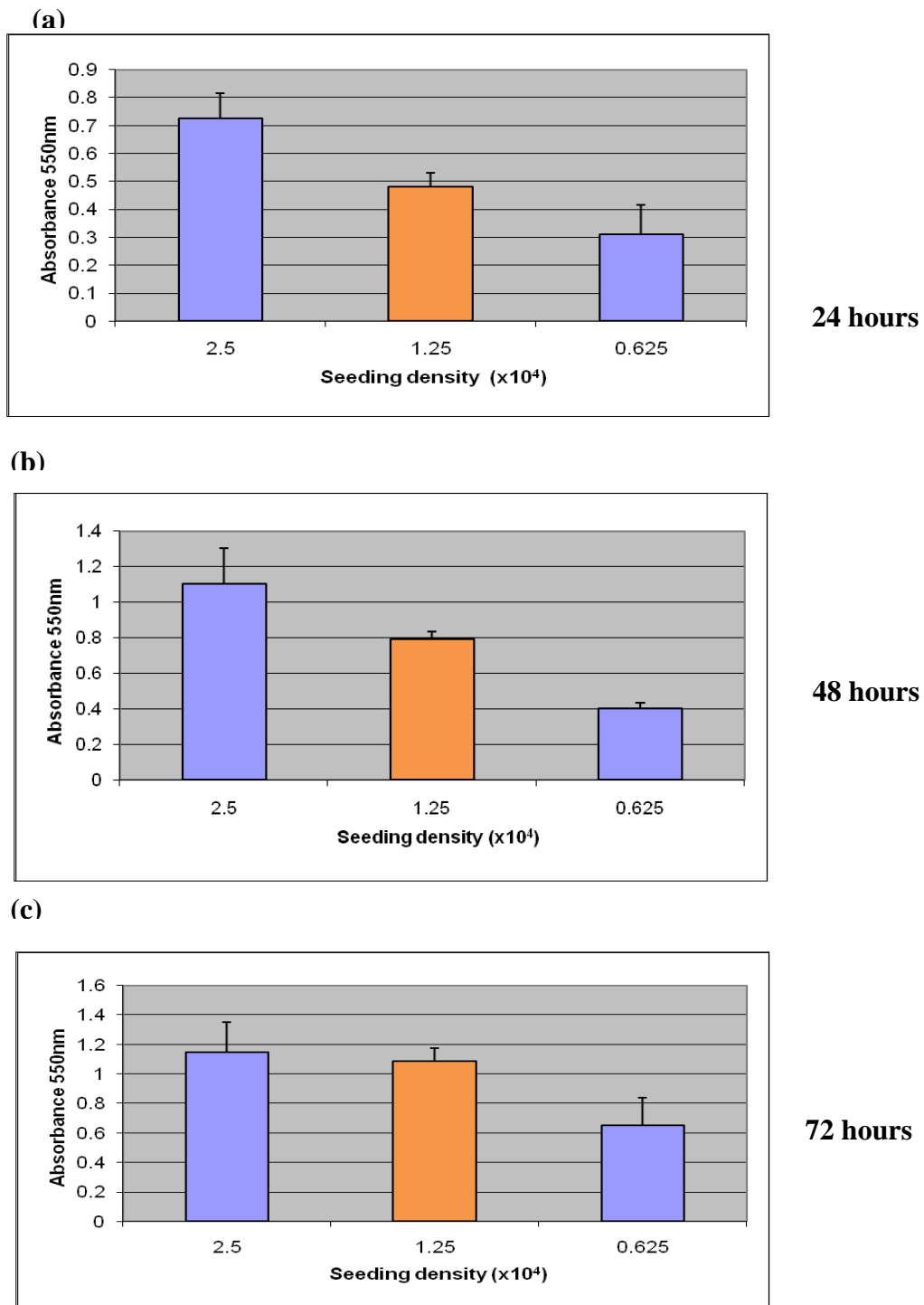


Figure 5.12: The selection of best seeding density and the time point for the dose response curve of different chemotherapeutic drugs treatment in JJ012 (NG2+ve) cells. Representative images for MTT results in untreated JJ012 (NG2+ve) cells. Each image represents pooled data from three independent experiments all done in triplicate. Cells were grown in culture for 24, 48 and 72 hours. The highlighted bars (orange colour) represent the seeding density chosen for the dose response curve.

5.2.2. The effect of chemotherapeutic drugs on JJ012(NG2+ve) and B3(NG2-ve) cells assessed by MTT and flow cytometry using annexin V staining

First, we tested the response of JJ012 (NG2+ve) cells to different concentrations of the chemotherapeutic drugs (doxorubicin, ifosfamide, docetaxel and cisplatin). These are the drugs which are used for treating patients with high grade sarcoma. To establish the IC₅₀ values for these chemotherapeutic drugs, JJ012(NG2+ve) cells were plated at 1.25×10^4 /well and treated with doxorubicin, ifosfamide, docetaxel and cisplatin for 48 hours at different doses (figure 5.13). Results showed that the IC₅₀ values can be calculated from the dose response curve of doxorubicin and docetaxel, which are 0.3 μ M and 10nm respectively, while results of cisplatin and ifosfamide dose response curves need to be further clarified, as there was a sudden decrease in the cell viability (demonstrated by sudden decline in the absorbance readings of the MTT assay) between 3 and 10mM doses of Ifosfamide and 10 and 30 μ M for Cisplatin.

Therefore, it was necessary to check why this sudden decrease in the cell viability happens with Ifosfamide and cisplatin doses. Do cells die from necrosis or apoptosis? Chemotherapeutic drugs work by inducing apoptosis in the cancer cells. Flow cytometry using annexin V staining was performed. Annexin V is used frequently in apoptosis studies, which can detect the translocation of phospholipids from inner to the outer cellular membrane during apoptosis. Flow cytometry results showed that when JJ012 (NG2+ve) cells pre-treated with 10mM Ifosfamide, the percentage of cells which undergo necrosis was much higher than the percentage of cells which undergo apoptosis (figure 5.14). However, both apoptosis and necrosis percentages were almost similar when JJ012 (NG2+ve) cells were pre-treated with

3mM Ifosfamide. This indicates that JJ12 cells are undergoing necrosis with little apoptosis induced by using these two doses of Ifosfamide.

By using cisplatin (10 and 30 μ M doses) to treat JJ012 (NG2+ve) cells, the percentage of cells undergo necrosis was higher than apoptosis at both doses (figure 5.14). The conclusion was that both drugs (Ifosfamide and cisplatin) would not be used as good models to study the role of NG2/CSPG4 in the chemoresistance of human chondrosarcoma

When JJ012 (NG2+ve) cells were treated with doxorubicin at 0.3 μ M and docetaxel 10nM, the percentage of cells that undergo apoptosis was higher than the percentage of cells that undergo necrosis (figure 5.15). Therefore, both drugs (doxorubicin and docetaxel) can be used to study the role of NG2/CSPG4 in the chemoresistance of human chondrosarcoma.

To further clarify the differential response of JJ012 (NG2+ve) and B3 (NG2-ve) cells to chemotherapeutic drugs (doxorubicin and docetaxel), MTT assays were undertaken on B3 (NG2-ve) cells following the same methodology used in JJ012 (NG2+ve) cells. Figure (5.16) shows the results of this experiment. Results showed that there was a difference in the cellular response to doxorubicin and docetaxel between JJ012 (NG2+ve) and B3 (NG2-ve) cells, which need to be clarified further. However, the difference was not statistically significant. Therefore, another experiment for detection of apoptosis by flow cytometry using annexin V was carried out (discussed in the next section).

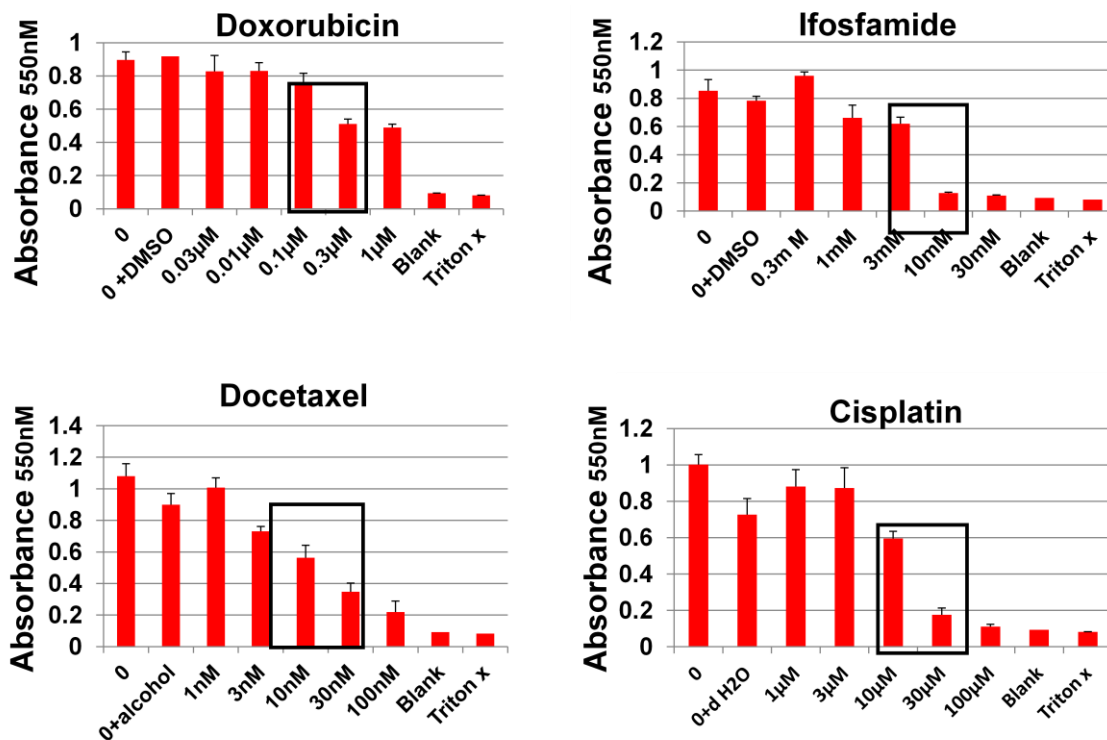


Figure 5.13: Dose response curves for JJ012 (NG2+ve) cells treated with different chemotherapeutic drugs (doxorubicin, ifosfamide, docetaxel and cisplatin) using MTT assay. Each image represents pooled data from 6 independent experiments, each done in triplicate. Cells were grown in culture for 48 hours and treated with different doses of chemotherapeutic drugs as illustrated and MTT was added. Readings were taken at 550nm absorbance. The highlighted bars (black colour) represent the estimated IC₅₀ value for each drug used. From the curves shown, it was possible to calculate the IC₅₀ value for doxorubicin and docetaxel, while results of ifosfamide and cisplatin curves showed a sudden decrease in the cell viability (demonstrated by a sudden decline in the absorbance readings of the MTT assay) between 3 and 10mM doses of Ifosfamide and 10 and 30μM for Cisplatin.

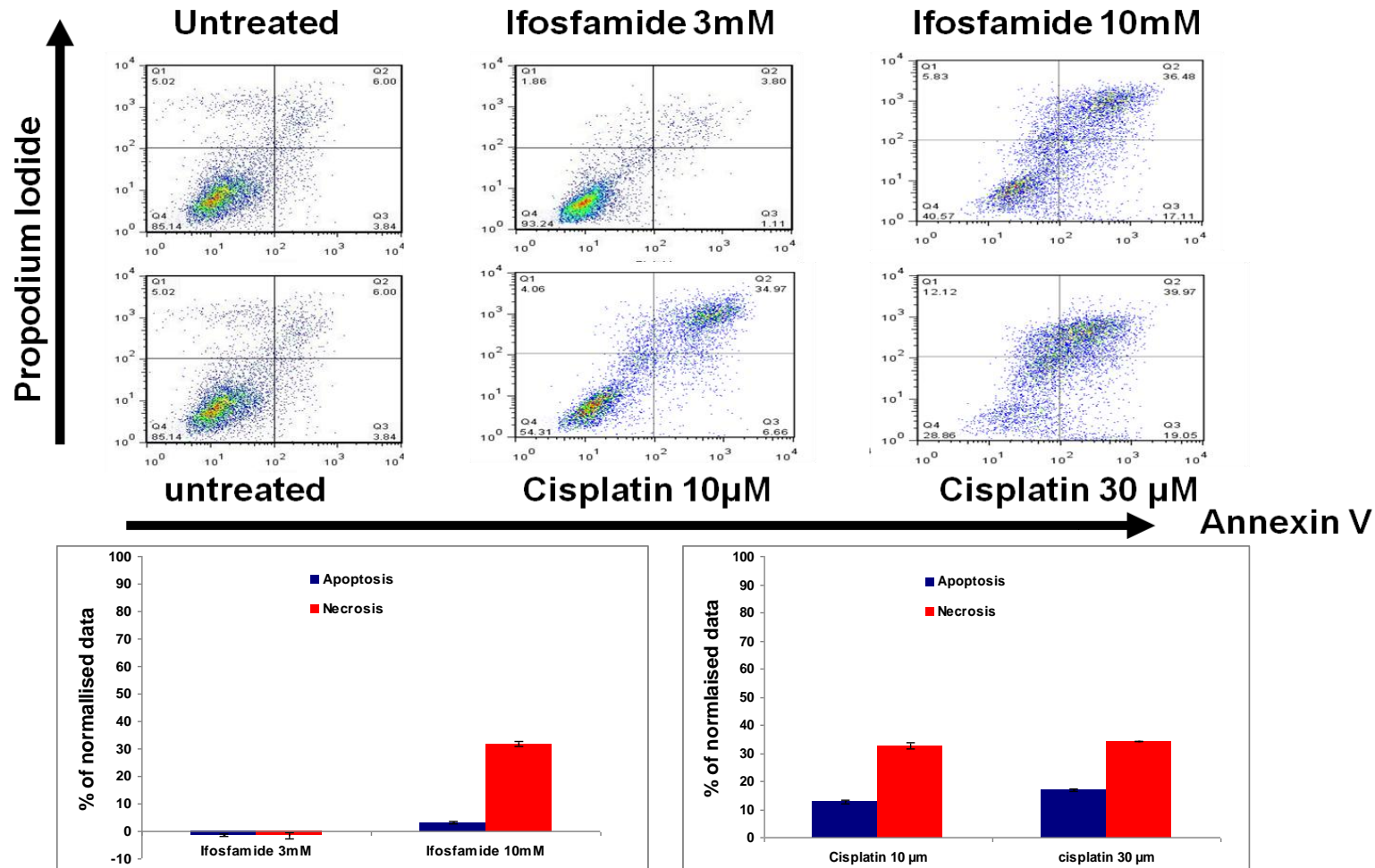


Figure 5.14: Testing the effect of chemotherapeutic drug (ifosfamide and cisplatin) on JJ012 (NG2+ve) cells. The figure shows representative images and graphs for apoptosis detection using annexin V staining flow cytometry. Each image represent pooled data from n=1 experiment, done in triplicate. Cells were grown in culture for 48 hours and treated with ifosfamide (10 and 30mM) and cisplatin (10 and 30µM). Cells were then passaged and stained with Annexin V. The graphs below show the percentages of apoptosis in treated cells normalised to untreated data. The percentages of cells undergo necrosis was more than apoptosis for both ifosfamide and cisplatin doses used in this experiment.

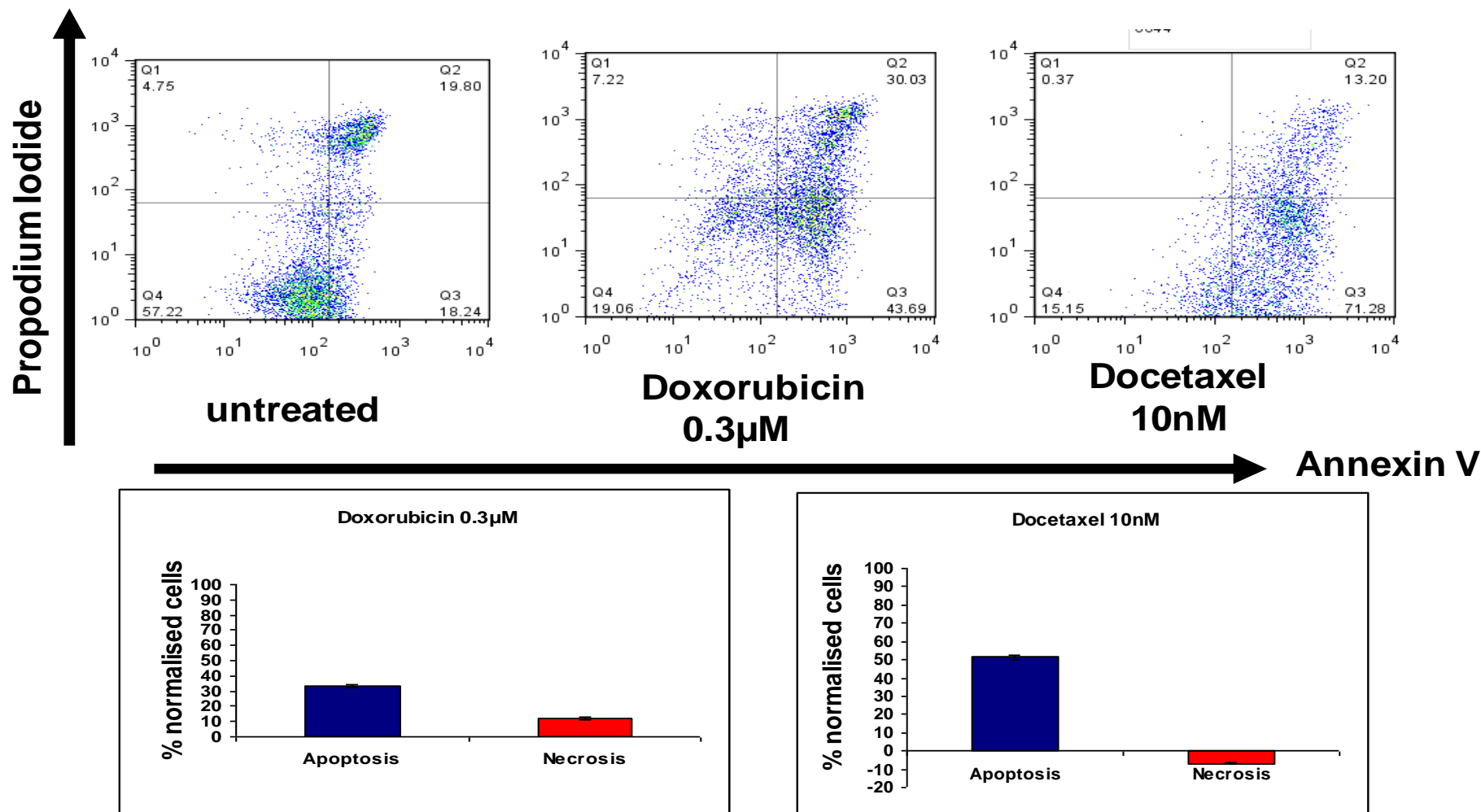


Figure 5.15: Testing the effect of chemotherapeutic drug (Doxorubicin and docetaxel) on JJ012 (NG2+ve) cells. Figure shows representative images and graphs for apoptosis detection using annexin V staining flow cytometry. Each image represent pooled data from n=1 experiment, each done in triplicate. Cells were grown in culture for 48 hours and treated with doxorubicin (0.3µM) and docetaxel (10nM). Cells were then passaged and stained with Annexin V. The graphs below show the percentages of apoptosis in treated cells normalised to untreated data. The percentage of cells that undergo apoptosis was higher than the percentage of cells that undergo necrosis for both doxorubicin and docetaxel doses used in this experiment.

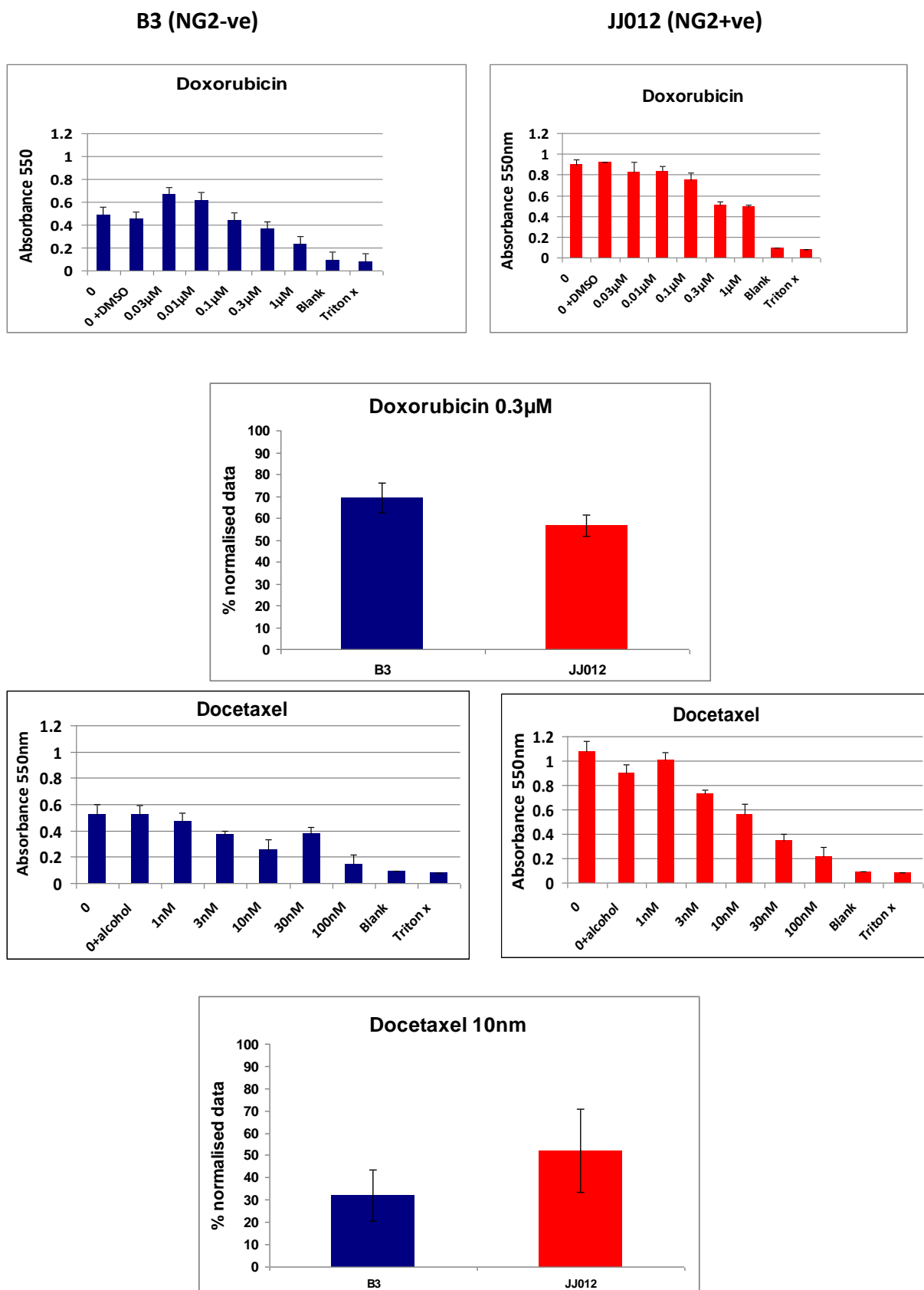


Figure 5.16: The differential response of JJ012 (NG2+ve) and B3 (NG2-ve) cells to different doses of doxorubicin and docetaxel pre-treatment. Cells were treated with different doses of doxorubicin and docetaxel as illustrated and MTT was carried out to assess the cellular response. The values of the estimated IC₅₀ value for doxorubicin (0.3µM) and docetaxel (10nM) were normalised to untreated values and presented in the graphs shown. Results represent pooled data from 6 independent experiments, each done in triplicate.

5.2.3 Detection of apoptosis by flow cytometry (Annexin V staining) at different time points in JJ012 (NG2+ve) and B3 (NG2 –ve) cells (24, 48 and 72 hours)

5.2.3.1 Detection of apoptosis by annexin V staining with 0.3 μ M doxorubicin for 24, 48 and 72 hours

JJ012 (NG2+ve), B3 (NG2-ve) together with non-target cells were grown in culture. Next day, cells were treated with 0.3 μ M doxorubicin for 24, 48 and 72 hours. At the end of each time point, cells were passaged and the percentage of apoptosis was assessed using annexin V staining. Statistical analysis was carried out using One-way ANOVA.

Results indicated that, when JJ012 (NG2+ve), B3 (NG2-ve) and non-target cells were treated with 0.3 μ M doxorubicin for 24 hours, there was no significant difference in the percentage of apoptosis between JJ012 (NG2+ve) and B3 (NG2-ve), with a P value= 0.4237. There was no difference in the percentage of apoptosis in JJ012 (NG2+ve) and the control cells (non-target) in this experiment. The data shown (figure 5.17 and 5.18) represents the average of three independent experiments+/-standard error of the mean (SEM).

For the 48 hours time point treatment, a statistically significant difference in the percentage of apoptosis was detected between JJ012 (NG2+ve) and B3 (NG2-ve) with less apoptosis percentage detected in JJ012 (NG2+ve) cells than in B3 (NG2-ve) cells. The P value was 0.0315. There was no difference in the percentage of apoptosis in JJ012 (NG2+ve) and the control cells (non-target) in this experiment. The data shown (figure 5.19 and 5.20) represents the average of ten independent experiments+/-standard error of the mean, each done in triplicate.

When the three cell types were treated with 0.3 μ M doxorubicin for 72 hours, there was approximately 25% of apoptosis detected in B3 (NG2-ve) cells, while JJ012 (NG2+ve) cells had a negative apoptosis percentage around 10%. The difference in the apoptosis percentage was not statistically significant between JJ012 and B3 cells, with a P value= 0.1021. There was also no difference in the percentage of apoptosis in JJ012 (NG2+ve) and the control cells (non-target) in this experiment. The data shown (figure 5.21 and 5.22) represents the average of three independent experiments \pm standard error of the mean, each done in triplicate.

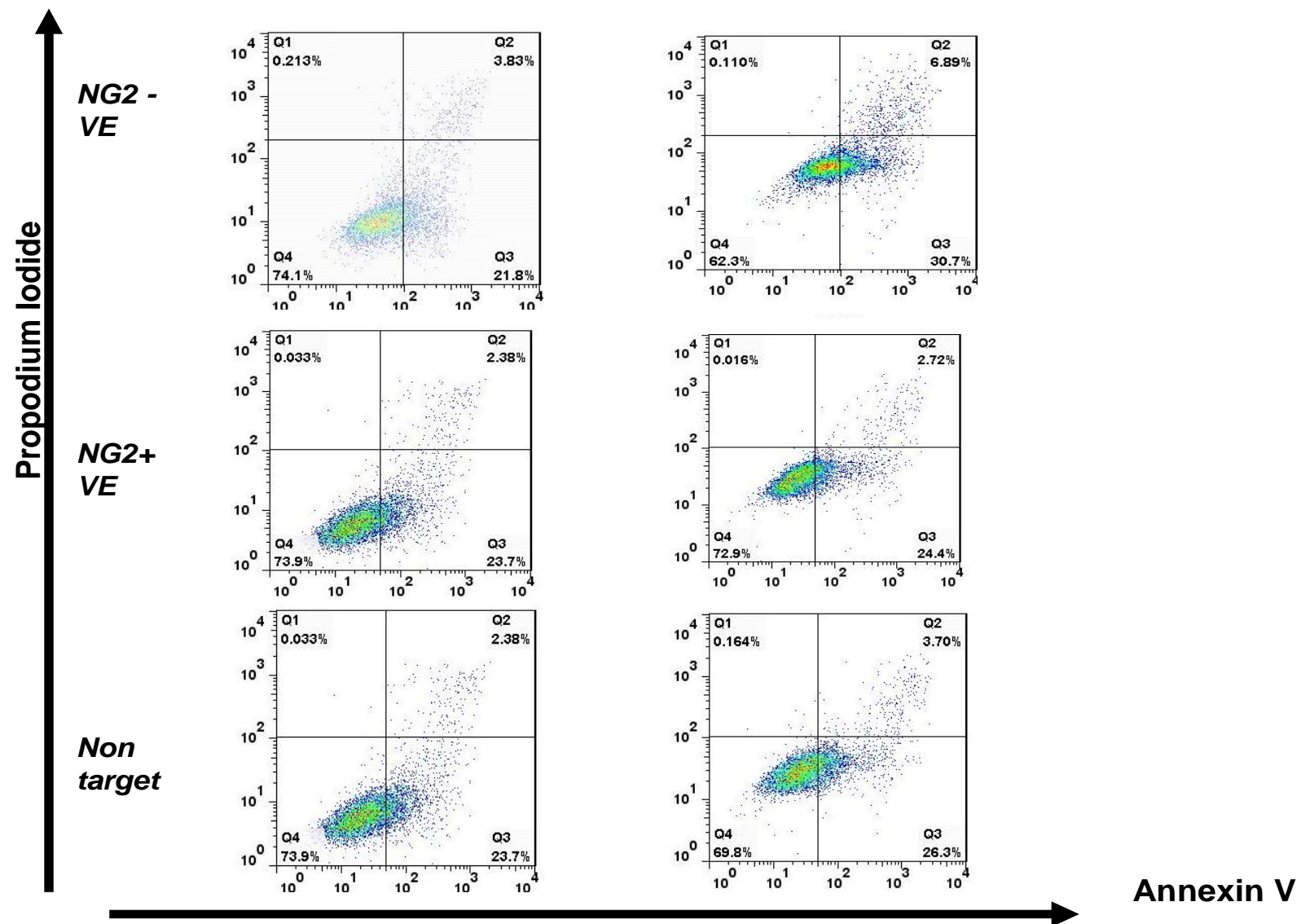


Figure 5.17: Representative image (n=3) for detection of apoptosis by flow cytometry using annexin V staining in JJ012 (NG2+ve) and B3 (NG2-ve) cells after 0.3µM doxorubicin treatment for 24 hours. Cells were grown in culture. Next day, cells were treated with 0.3µM doxorubicin for 24 hours, passaged and stained with annexin V. There was no statistically significant difference in the percentage of apoptosis between both cell types, with a P value = 0.4237(not significant).

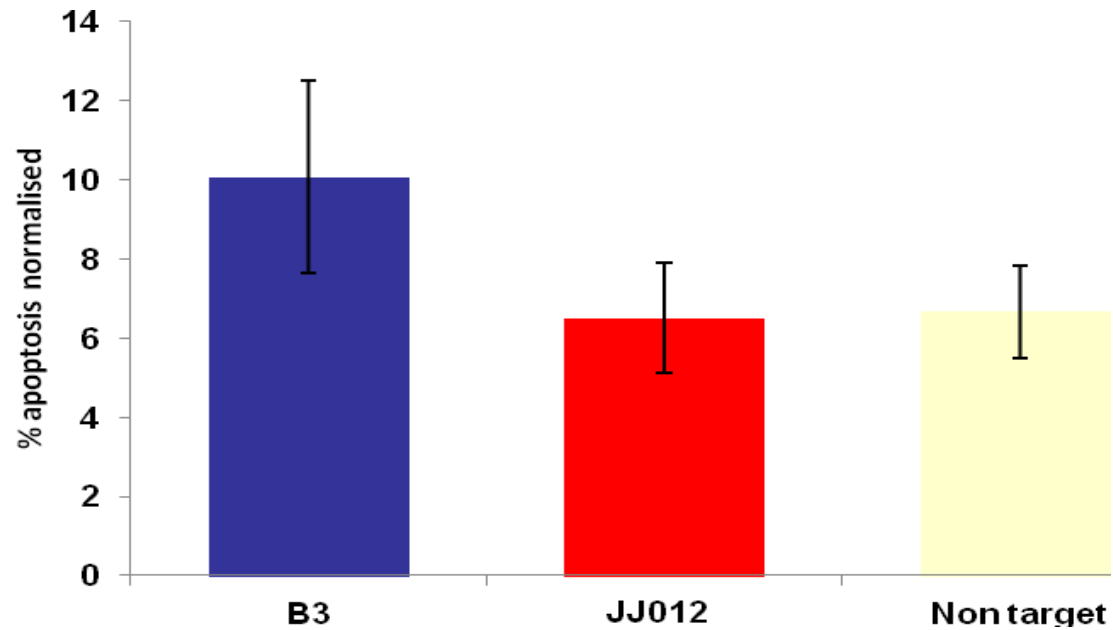


Figure 5.18: No significant difference in the apoptosis percentage was detected between JJ012 (NG2+ve) and B3 (NG2-ve) after treatment with 0.3 μ M doxorubicin for 24 hours. Cells were grown in culture. Next day, cells were treated with 0.3 μ M doxorubicin for 24 hours, passaged and stained with annexin V. The percentages of apoptosis shown were normalised to untreated data. Results represent the pooled data of three independent experiments, each carried out in triplicate \pm SEM, with a P value = 0.4237(not significant).

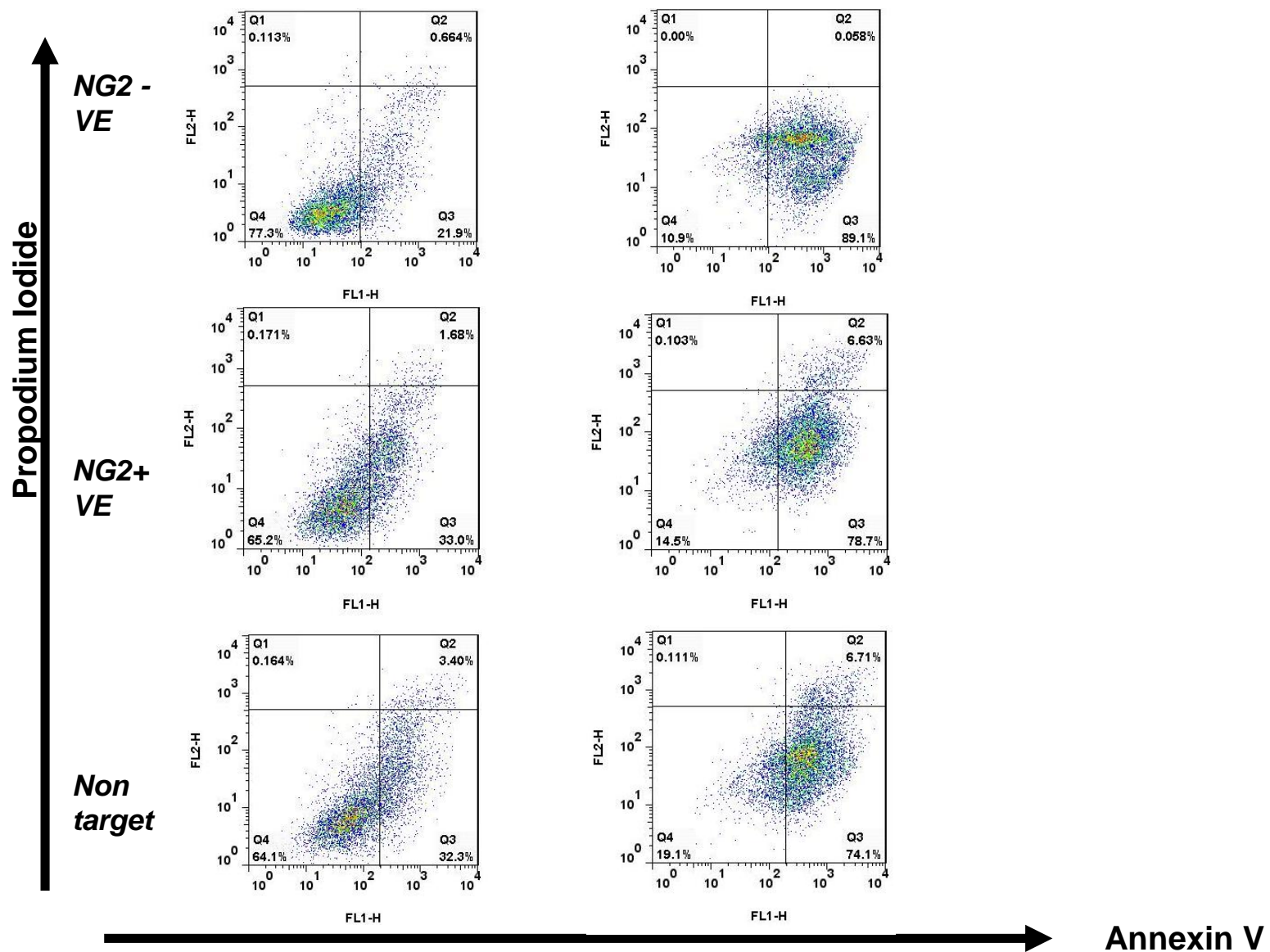


Figure 5.19: Representative images (n=10) for the apoptosis detection by flow cytometry using annexin V staining in JJ012 (NG2+ve) and B3 (NG2-ve) cells after 0.3 μ M doxorubicin treatment for 48 hours. Cells were grown in culture. Next day, cells were treated with 0.3 μ M doxorubicin for 48 hours, passaged and stained with annexin V. There was a statistically significant difference in the apoptosis percentage between both cell types, with a P value=0.0315(significant).

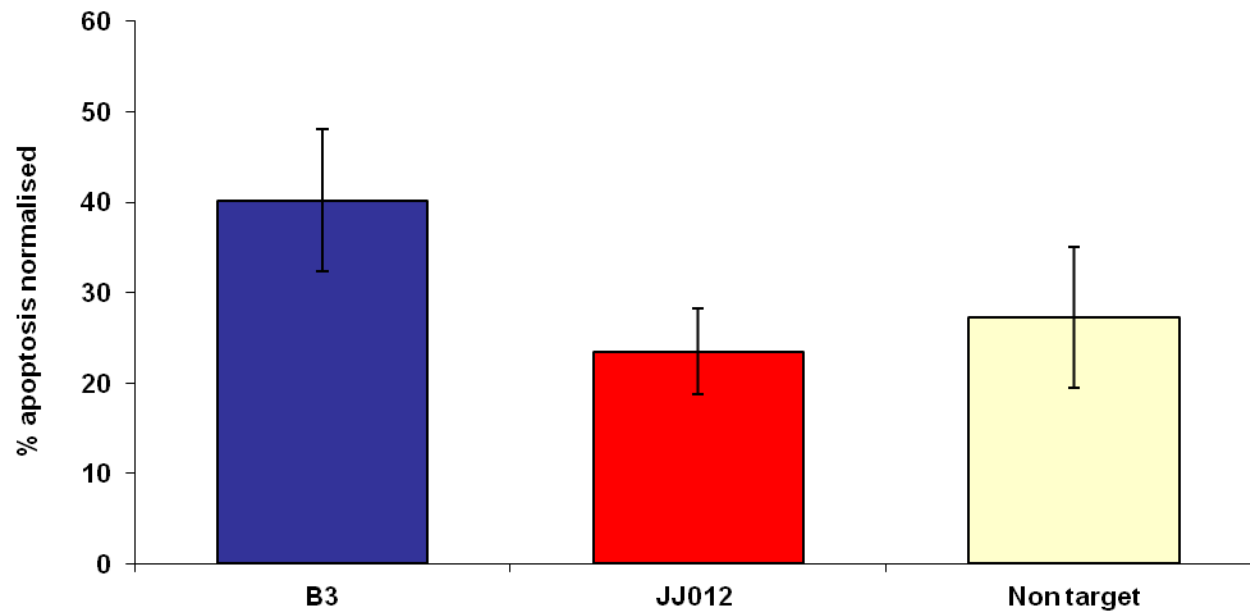


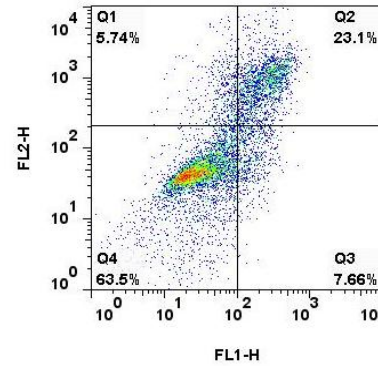
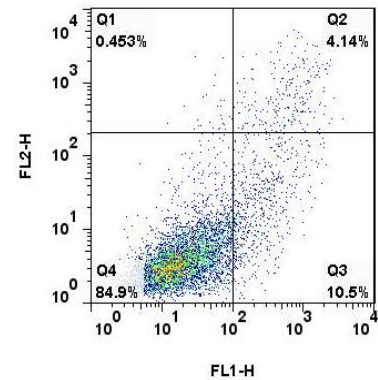
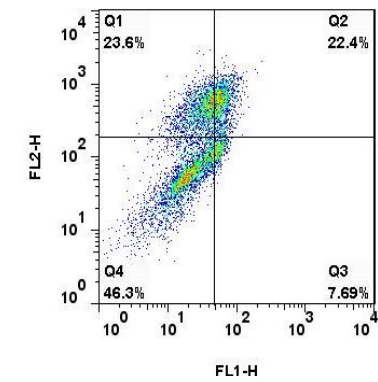
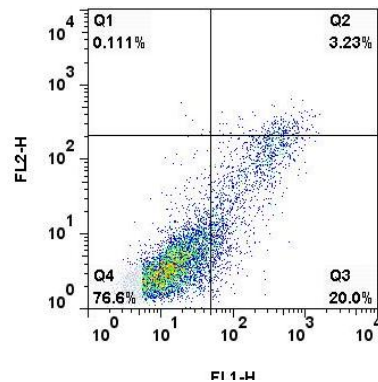
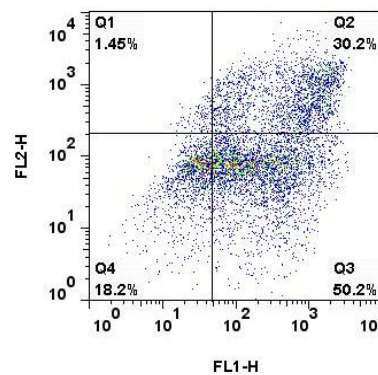
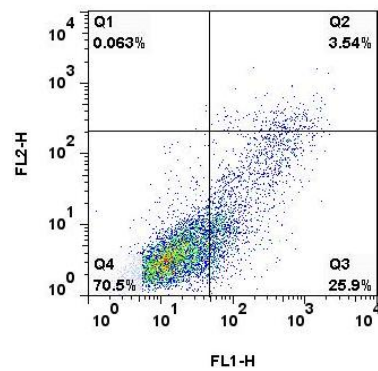
Figure 5.20: Significant difference in the apoptosis percentage was detected between JJ012 (NG2+ve) and B3 (NG2-ve) cells after 0.3 μ M doxorubicin treatment for 48 hours. Cells were grown in culture. Next day, cells were treated with 0.3 μ M doxorubicin for 48 hours, passaged and stained with annexin V. The percentages of apoptosis shown were normalised to untreated cells. Results represent the pooled data of ten independent experiments, each carried out in triplicate \pm SEM, with a P value=0.0315 (significant).

Propidium Iodide

NG2 -
VE

NG2+
VE

Non
target



Annexin V

Figure 5.21: Representative images (n=3) for the apoptosis detection by flow cytometry using annexin V staining in JJ012 (NG2+ve) and B3 (NG2-ve) cells after 0.3 μ M doxorubicin treatment for 72 hours. Cells were grown in culture. Next day, cells were treated with 0.3 μ M doxorubicin for 72 hours, passaged and stained with annexin V. There was no statistically significant difference in the apoptosis percentages between both cell types, with a P value=0.1021(not significant).

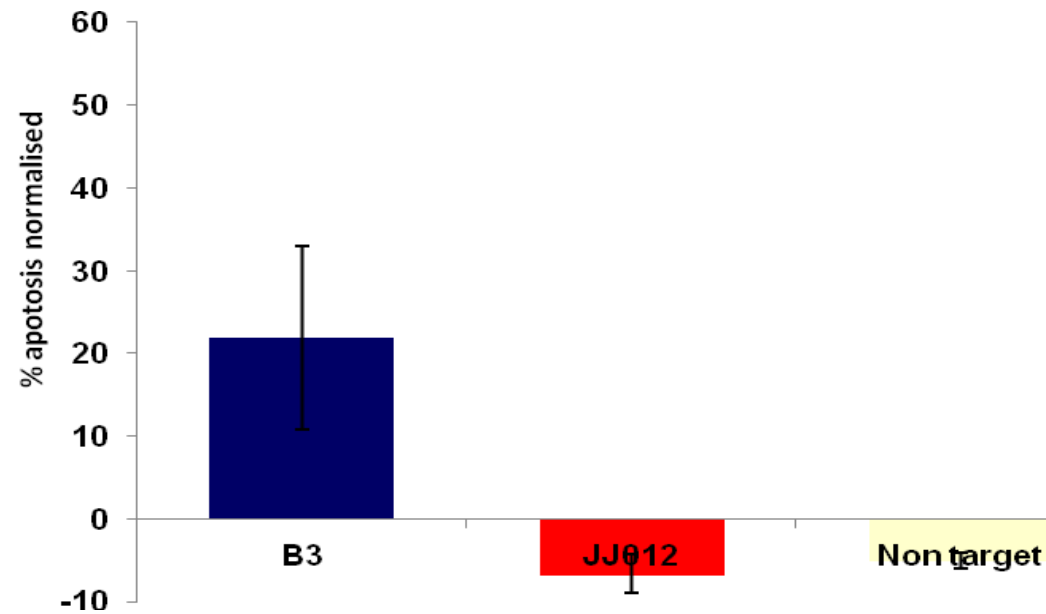


Figure 5.22: No significant difference in the apoptosis percentage was detected between JJ012 (NG2+ve) and B3 (NG2-ve) cells after 0.3 μ M doxorubicin for 72 hours. Cells were grown in culture for 48 hours, treated with 0.3 μ M doxorubicin for 72 hours, passaged and stained with annexin V. The apoptosis percentages shown were normalised to untreated cells. Results represent the pooled data of ten independent experiments, each carried out in triplicate \pm SEM, with a P value=0.1021 (not significant).

5.2.3.2 Detection of apoptosis by annexin V staining with 10nm Docetaxel treatment for 48 hours

JJ012 (NG2+ve), B3 (NG2-ve) together with non-target cells were grown in culture. The next day the cells were treated with 10nm docetaxel for 48 hours. At the end of each time point, cells were passaged and the rate of apoptosis was assessed using annexin V staining.

Results indicated that, when JJ012 (NG2+ve), B3 (NG2-ve) and non-target cells were treated with 10nm docetaxel for 48 hours, there was no significant difference in the apoptosis percentage between JJ012 (NG2+ve) and B3 (NG2-ve) cells. There was no difference in the percentage of apoptosis in JJ012 (NG2+ve) and the control cells (non-target) in this experiment. The data shown (figure 5.23 and 5.24) represents the average of three independent experiments \pm standard error of the mean (SEM).

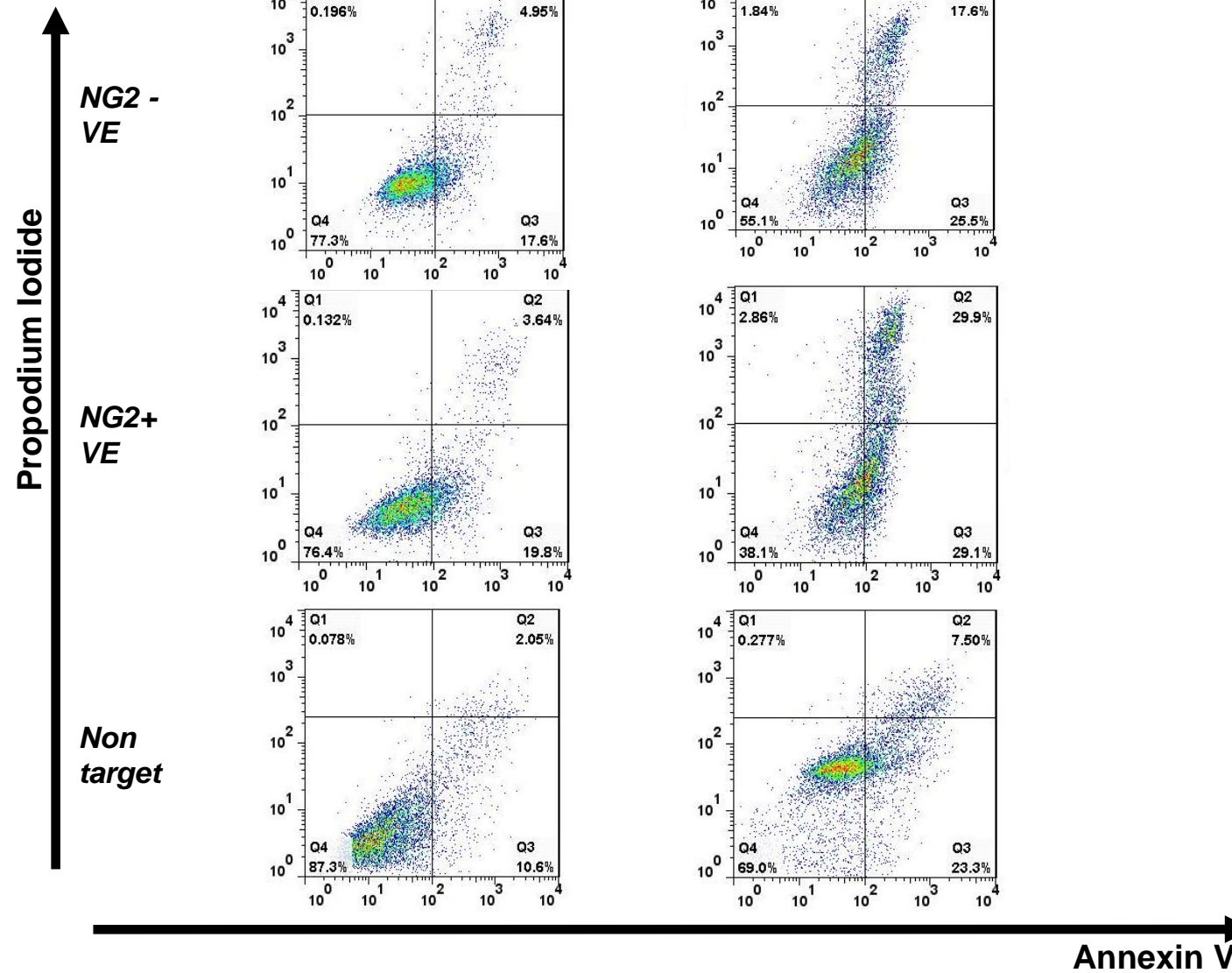


Figure 5.23: No Significant difference in the apoptosis percentage between JJ012 (NG2+ve) and B3 (NG2-ve) cells after 48 hours 10nm Docetaxel treatment. Cells were grown in culture for 48 hours, treated with 10nm docetaxel for 48 hours, passaged and stained with annexin V. The apoptosis percentages shown were normalised to untreated cells. Results shown are the percentages of apoptosis of three independent experiments, each carried out in triplicate \pm SEM.

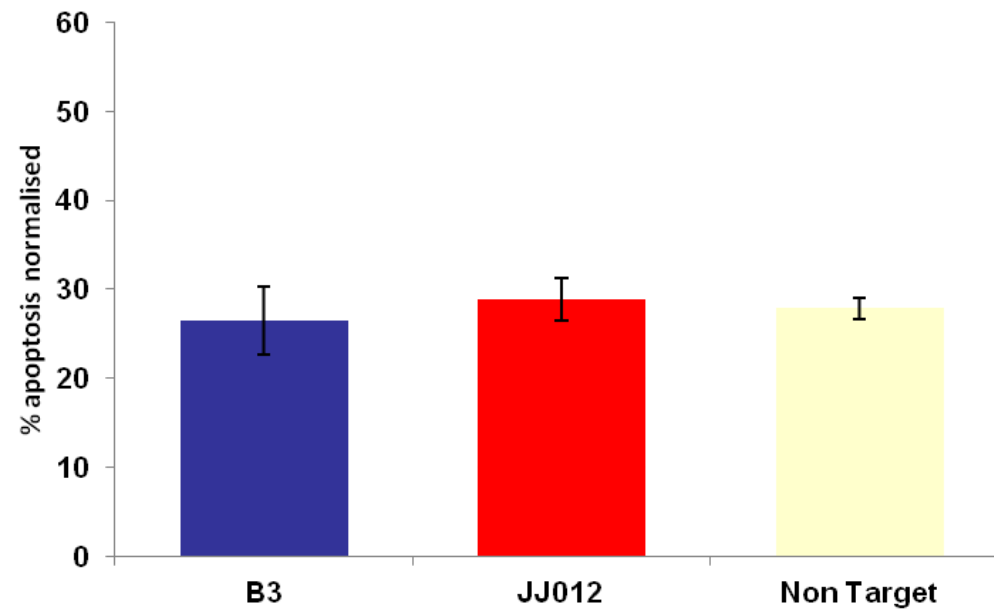


Figure 5.24: No significant difference in the apoptosis percentage between JJ012 (NG2+ve) and B3 (NG2-ve) cells after 48 hours 10nm docetaxel treatment. Cells were grown in culture for 48 hours, treated with 10nm docetaxel for 48 hours, passaged and stained with annexin V. The apoptosis percentages shown were normalised to untreated cells. Results shown are the percentages of apoptosis of three independent experiments, each carried out in triplicate \pm SEM.

5.4 The effect of NG2/CSPG4 knockdown on chondrocyte gene expression

I investigated the effect of NG2/CSPG4 loss on a range of cellular genes: 1) cartilage matrix molecules and transcription factors markers (Collagen II, Aggrecan, Decorin, Sox-9 and Run-x2); 2) Genes involved in cartilage degeneration in OA (MMP3, MMP13, ADAMTS-4 and 5); 3) Pro and anti-inflammatory cytokines involved in OA process (IL1 β , IL2R γ , and IL4R, IL10 and IL13Ralpha1 and 2); 4) ER stress markers (HSP70, GRP8, PDI and Bag-1); and 5) Genes for the known extracellular ligands of NG2/CSPG4 (Collagen VI, PDGFA and B, TIMP3 and FGF2).

The results showed that, following NG2 knockdown in JJ012 cells there was no difference in expression of aggrecan, collagen II and SOX-9. In contrast cells in which NG2/CSPG4 had been knocked down showed decreased expression of MMP3 and ADAMTS-4, a complete loss of ADAMTS-5 and increased expression of MMP13. Figure 5.25a shows the results of this experiment.

The effect of NG2 knockdown on the pro and anti-inflammatory cytokines involved in OA process was differential. While there was no effect of NG2 knockdown on expression of IL-1 β , the main pro-inflammatory cytokine involved in OA process, there was a reduced expression of the anti-inflammatory cytokine, IL10, in B3(NG2-ve) cells in comparison to JJ012(NG2+ve) cells (figure 5.25b). There was an increased expression of IL2R γ receptor in B3 (NG2-ve) cells, with no difference in the expression level of IL13R alpha1 and 2 and IL4R between B3 (NG2-ve) cells and JJ012 (NG2+ve) cells (figure 5.25c). However, further studies are required to assess

the effect of NG2/CSPG4 knockdown on the cytokine and its matching cell surface receptor to further support these data.

The effect of NG2/CSPG4 knockdown on ER stress marker was also differential. In figure (5.25d), the results showed that there was no effect of NG2/CSPG4 knockdown on HSP70, GRP8 and Bag-1, with increased expression of PDI in B3(NG2-ve) cells.

I investigated the effect of NG2/CSPG4 loss on its extracellular ligands in JJ012 chondrosarcoma cells. There was no effect on the expression level of collagen VI and FGF-2. A complete loss of the expression of PDGFA, with increased expression of PDGFB in NG2 knocked down cells was observed. Figure (5.25e) shows the results of this experiment.

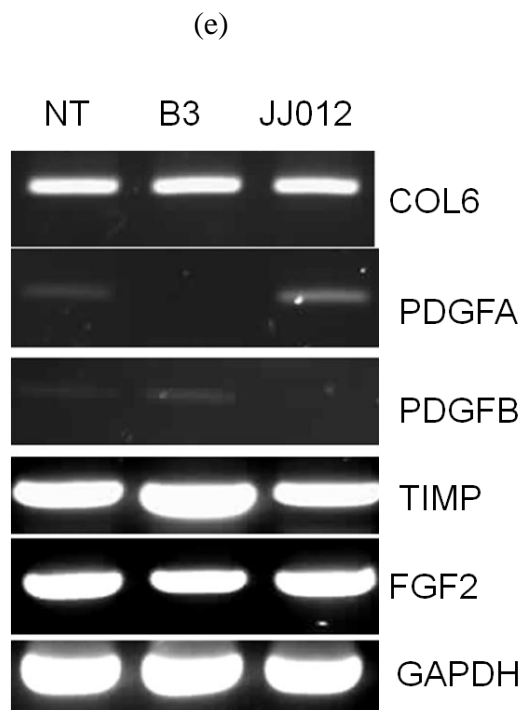
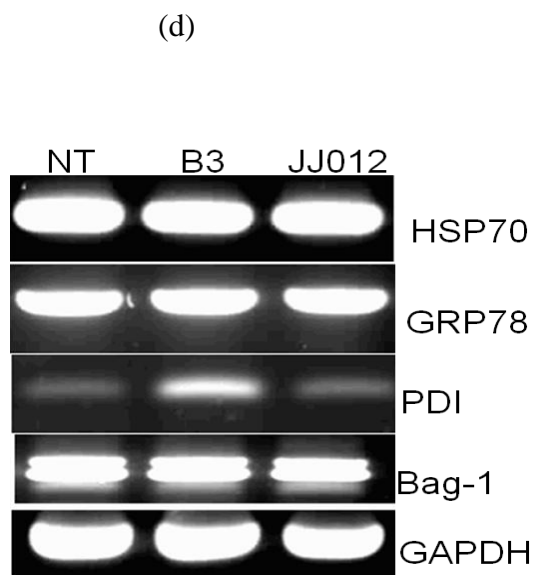
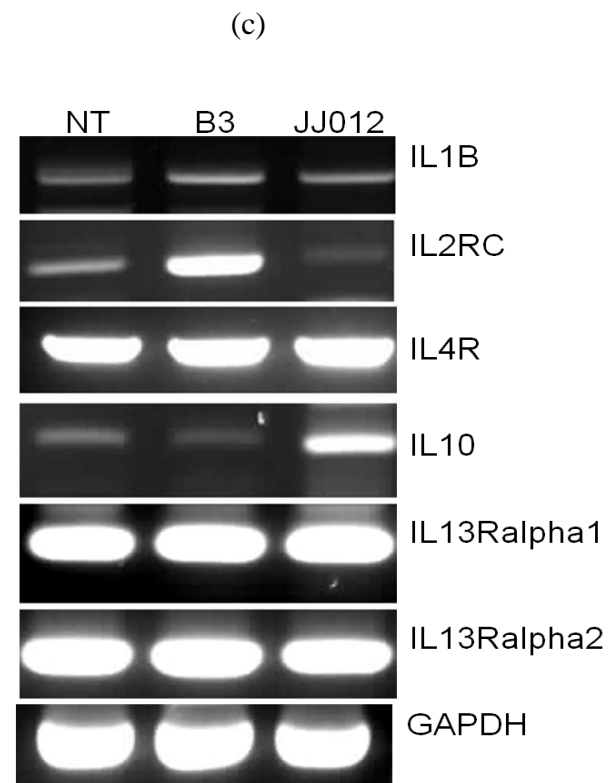
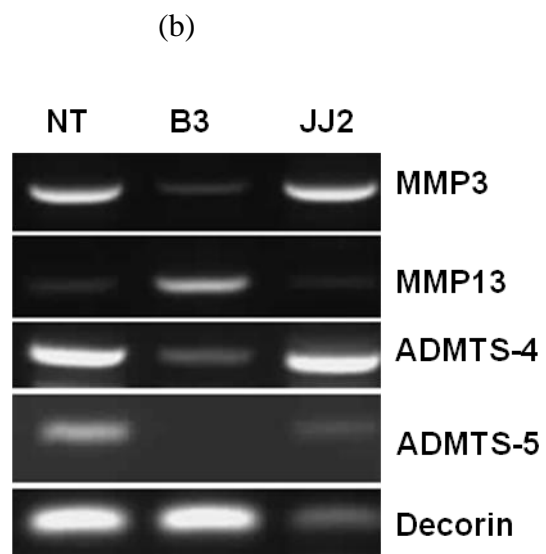
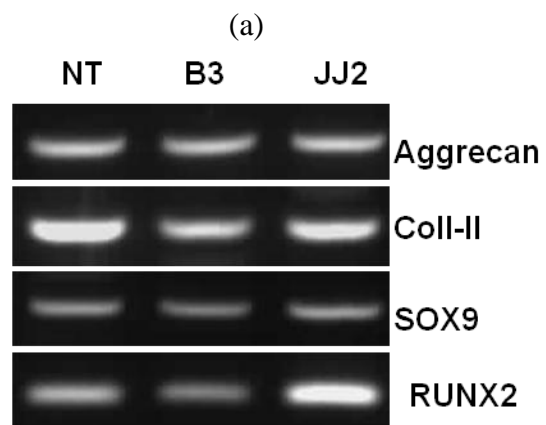


Figure 5.25: Representative images of RT-PCR reaction for the effect of NG2/CSPG4 knockdown on different cellular genes. Although in figure (a), there was no difference in expression of aggrecan, collagen II and SOX-9 in both JJ012(NG2+ve) and B3 (NG2-ve) cells, figure (b) showed a decreased expression of MMP3 and ADAMTS-4, a complete loss of ADAMTS-5 and increased expression of MMP13 in B3 (NG2-ve) cells. The effect of NG2/CSPG4 knockdown on the pro and anti-inflammatory cytokines involved in OA process was differential (figure (c)): 1) No effect of NG2 knockdown on expression of IL-1.2) A reduced expression of the anti-inflammatory cytokine, IL10 in B3 (NG2-ve) cells in comparison to JJ012 (NG2+ve) cells. 3) There was an increased expression of IL2RG receptor in B3 (NG2-ve) cells. 4).No difference in the expression level of IL13R alpha1 and 2 and IL4R between B3 (NG2-ve) cells and JJ012 (NG2+ve) cells. In figure (d) (the effect of NG2/CSPG4 knockdown on the ER stress markers) there was no effect of NG2/CSPG4 knockdown on HSP70, GRP8 and Bag-1, with increased expression of PDI in B3 (NG2-ve) cells. Figure (e) showed the effect of NG2/CSPG4 loss on its extracellular ligands in JJ012 chondrosarcoma cells. There was no effect on the expression level of expression of collagen VI and FGF-2, a complete loss of the expression of PDGFA, with increased expression of PDGFB in NG2 knocked down cells was observed.

5.5 Summary

B3 cells spread more and migrate less than JJ012 cells; with a significant difference observed in migration (after 10 hours: the closed area was 81.4% for JJ012 and 54.6% for B3). There was no difference in cell adhesion to collagen I, II, VI and fibronectin. EGTA inhibited cell adhesion to fibronectin in dose dependent manner with no significant difference observed between both JJ012 and B3 cells. EDTA reduced adhesion of B3 cells but not JJ012 to fibronectin. A significant difference in cell proliferation was detected, with no change in the apoptosis percentages in both cell types at all time points. A significant difference in the apoptosis percentage was observed in B3 (NG2-ve) cells in comparison to JJ012 (NG2 +ve) cells after Doxorubicin treatment for 48 hours.

Following NG2/CSPG4 knockdown in JJ012 cells there was no difference in expression of aggrecan, collagen II and SOX-9. Contrast cells in which NG2/CSPG4 had been knocked down showed decreased expression of MPP3 and ADAMTS-4, a complete loss of ADAMTS-5 and increased expression of MMP13. The effects of NG2/CSPG4 knockdown on the pro and anti-inflammatory cytokines involved in OA process were differential: 1) No effect of NG2 knockdown on expression of IL-1. 2) A reduced expression of the anti-inflammatory cytokine, IL10 in B3 (NG2-ve) cells in comparison to JJ012 (NG2+ve) cells. 3) There was an increased expression of IL2RG receptor in B3 (NG2-ve) cells. 4) No difference in the expression level of IL13R alpha1 and 2 and IL4R between B3 (NG2-ve) cells and JJ012 (NG2+ve) cells. For ER stress markers, there was no effect of NG2/CSPG4 knockdown on HSP70, GRP8 and Bag-1, with increased expression of PDI in B3 (NG2-ve) cells. In addition, results for the effect of NG2/CSPG4 knockdown on its extracellular ligands showed that

there was no effect on the expression level of expression of collagen VI and FGF-2. A complete loss of the expression of PDGFA, with increased expression of PDGFB in NG2 knocked down cells was observed.

5.6 Discussion

5.6.1 The role of NG2/CSPG4 in cell adhesion, spreading, migration, proliferation and invasion in human chondrocytes

5.6.1.1 NG2/CSPG4 and cell adhesion

Different studies highlighted the involvement of NG2/CSPG4 in the cell adhesion, spreading, migration, invasion and proliferation. It is well known that the adhesion and migration processes are regulated by different membrane proteins, where integrins represents the main molecule in this process (Humphries 2000). However, NG2/CSPG4 is also able to modulate these processes. An earlier study showed that, in melanoma cells, NG2/CSPG4 is localised at specific microdomains, microspikes, where it may be involved in the cell adhesion process (Garrigues et al. 1986).

Another study showed that NG2/CSPG4 negatively modulates the adhesion, migration and the potentials of human melanoma cells. NG2/CSPG4 was found to be associated with two functionally important ligands: CD44 and $\alpha 4\beta 1$ integrin. Transfection of rat NG2 into the NG2-negative B16 mouse melanoma cell also resulted in the same association. However, the expression of NG2/CSPG4 decreased the adhesion of B16 melanoma cells to CD44 monoclonal antibodies, hyaluronic acid, the C-terminal 40-kDa fibronectin fragment, and the CS1 fibronectin peptide, increased the proliferation of melanoma cells in culture and increased tumorigenicity *in vivo*. Moreover, NG2/CSPG4 expression increased lung metastasis of B16F1 and B16F10 melanoma cells in experimental metastasis studies (Burg et al. 1998).

In brain tissues, it has been found that several brain CSPGs, including NG2/CSPG4, interact with proteins in the extracellular matrix and with receptors on neural cells. Different studies explained in a review paper, published by Grumet and his colleagues in 1996, demonstrated that the brain CSPGs can promote transient adhesion of neuronal cells, and inhibit stable cell adhesion and neurite growth, promoted by the cell adhesion molecules (Grumet et al. 1996). When expressed in regions containing low levels of adhesion molecules, various CSPGs including NG2 proteoglycan may act as barriers to cell migration and axonal growth, whereas in the regions with high levels of adhesion proteins, brain CSPGs may still act to maintain certain boundaries while allowing selective axonal extension to proceed. It appears that the relative levels of these molecules as well as the organisation of the extracellular matrix may be important factors that regulate the rate of axonal growth locally. Also, the differential expression of CSPGs may be important for modulating cell adhesion as well as axonal growth and guidance during neural development. However, the persistent expression of these molecules may prevent these processes in the normal mature nervous system as well as following brain injury (Grumet et al. 1996).

Research done in 2004 showed the importance of NG2/CSPG4 in the cell adhesion and its significant implication as a therapeutic target. In this work, it has been found that induction of humoral anti-human high molecular weight melanoma-associated antigen (anti-HMW-MAA) immunity, following active specific immunotherapy, is associated with a statistically significant prolongation of survival in patients with melanoma. This association does not appear to be mediated by immunological mechanism. However, it has been found that the non-immunological mechanisms by which anti-HMW-MAA antibodies (Abs) affect melanoma cells is through the interaction of HMW-MAA with extracellular matrix (ECM) components and HMW-MAA mAbs appear to block the series of signal transduction events

triggered by the interaction of HMW-MAA with ECM. They include the activation of the family of Rho GTPases, p130cas, and focal adhesion kinase (FAK) (Campoli et al. 2004; Chang et al. 2004).

In this study, when JJ012 (NG2+ve) cells and B3 (NG2-ve) cells were treated with $\alpha V\beta 3$ integrin blocking antibody, no significant effect on the cell adhesion process was observed in either cell types. Therefore, it appears that $\alpha V\beta 3$ integrin **is not** involved in the adhesion mechanism in human chondrosarcoma cells. However, by using $\beta 1$ integrin blocking antibodies, there was a significant reduction in cell adhesion in JJ012 (NG2+ve) cells in comparison to B3 (NG2+ve) cells when cells were grown on coated wells with collagen II, VI and FN. As explained in chapter five (results), when both cell types were treated with EDTA (which has low affinity for calcium and low affinity for magnesium) and EGTA (which preferentially binds to calcium rather than other cations, with a significantly greater affinity for calcium compared to EDTA), there was a reduction in cell adhesion in a dose dependent manner with EGTA pre-treatment in both cell types. By contrast EDTA has a reduced adhesion of B3 (NG2-ve) cells but not JJ012 (NG2+ve) cells. Therefore, from the data obtained, it might be possible to conclude that B3 (NG2-ve) cells relied on other molecules of cell adhesion to compensate for the loss of NG2/CSPG4 and NG2/ CSPG4 is indirectly involved in the cell adhesion process.

Cell adhesion molecules are proteins located on the cell surface and they are classified into calcium dependent (such as integrins, cadherins and selectins) and calcium independent cell adhesion (such as the immunoglobulin super family). Although several proteins are involved in the cell adhesion process, it is well known that the integrins family are unique in that they possess a complex structure, widely distributed in different tissues, as well as their activities

being regulated by inside-outside signalling (Gahmberg et al. 2009). Larger number of articles discussed the role of integrins in cell adhesion process. The binding of ligands to integrins is cross linked by divalent cations. The β -propeller domain of α subunit contains three or four β -hairpin loop-like Ca (2+)-binding motifs that have essential roles in integrin biogenesis. The function of another Ca (2+)-binding motif located at the region of α subunit remains elusive (Zhang and Chen 2012).

It has been shown that NG2/CSPG4 may be indirectly involved in cell adhesion, by acting as co-receptors for integrins. Iida and her colleagues in 1995 identified that, in melanoma cells, there was an increase in the spreading and focal contacts formations in response to both NG2/CSPG4 and $\alpha 4\beta 1$ integrin coated substrata (Iida et al. 1995). While melanoma cells adhere only, without spreading or focal contact formation when grown on either NG2/CSPG4 or $\alpha 4\beta 1$ integrin coated substrata alone. This indicated that collaboration of the two adhesion receptors (NG2/CSPG4 and $\alpha 4\beta 1$ integrin) change the adhesion ability of these cells, enabling them recognise the cytoskeleton and activate another cellular process (spreading) (Iida et al. 1995).

It has also been found that in human normal articular chondrocytes, the adhesion process to different ECM matrices (collagen II, VI and FN) is dependent on $\beta 1$ integrin. When cells were stimulated with NG2 antibodies, they become more adhesive to type VI collagen rather than other ECM matrices (Midwood and Salter 2001). This indicates that, as well as NG2 acting as a co-receptor for $\beta 1$ integrin, the characterisation of collagen VI as a cell surface receptor for NG2/CSPG4.

NG2/CSPG4 may also mediate cell adhesion and activate other cellular events independent of $\beta 1$ integrin. Added to this, NG2/CSPG4 possesses a differential response to different extracellular matrices. Heterogeneous expression of NG2/CSPG4 in the GD25 cell line (do not express $\beta 1$ integrin) enhances the cells' adhesion to collagen VI and V coated surfaces, but not to other collagens and laminin. More interestingly, the adhesion of GD25 cells to collagen VI triggers the cells spreading by the formation of ruffling lamellipodia, while these events were not observed when cells were grown on collagen V coated surfaces. This happened despite the fact that both collagens: type VI and V bind to the same central non-globular domain of NG2/CSPG4 (Tillet et al. 2002).

It has been found that NG2/CSPG4 is involved in the cell adhesion and even cell spreading indirectly, using special signal transduction pathways. It has been found that, in melanoma cells, melanoma chondroitin sulphate proteoglycan (MCSP) stimulates $\alpha 4 \beta 1$ integrin, which results in increasing cell adhesion and spreading of melanoma cells. MCSP activates Cdc-42 to its GTP-bound form. Cdc-42 is recognised by the (activated Cdc-42-associated kinase), resulting phosphorylation of the tyrosine residue on p130 and stimulation of the $\alpha 4 \beta 1$ integrin (Eisenmann et al. 1999). The use of specific inhibitors to Cdc-42 and p130 inhibits the activity of MCSP, indicating the importance of MCSP in this cellular process.

5.6.1.2 NG2/CSPG4 and cell spreading

We found that the loss of NG2/CSPG4 from JJ012 (NG2+ve) chondrosarcoma cells increased the cell spreading process. Bright field microscope showed that JJ012 (NG2+ve) cells were still rounded when grown on FN coated surface, while B3 (NG2-ve) cells showed the presence of lamellopodia and filopodia on the cell surface. One of the explanations for this is that JJ012 (NG2+ve) cells may be preparing themselves for the process of cell

division, which is supported by other data in this study. We found that JJ012 (NG2+ve) cells proliferate more than B3 (NG2-ve) cells. Added to this, it is known that the migration process is considered as a multi-step process, involving adhesion disassembly and retraction fibres formation (Ridley et al. 2003). Using wound scratch migration assay, JJ012 (NG2+ve) cells migrate more than B3 (NG2 -ve) cells.

Changes in the actin cytoskeleton are the main processes happening in both rounded and well spreading JJ012 cells. It seems to be that, in this work, the presence or absence of NG2 would trigger changes in the actin cytoskeleton. However, from previous studies, it is well known that NG2 is associated with different types of actin on both spreading and rounded cells. On the surfaces of the cells with flattened morphology, NG2/CSPG4 is present on the cell surface in linear arrays, which are aligned with actin- and myosin-containing stress fibres in the cytoskeleton. This unique distribution of NG2/CSPG4 can be disrupted by cytochalasin D, which also leads to the loss of these micro-filamentous stress fibres. In addition to the linear distribution of NG2/CSPG4 on the surface of flattened cells, NG2/CSPG4 can be present on the long, delicate tendrils extending from the periphery of cells that had lost their flattened morphology. In fact, these cells were rounded before, either in preparation for cell division or due to changes in the local adhesive environment (Lin et al. 1996). The tendrils observed in NG2 positive cells appear to be similar to the so called retraction fibres previously observed in association with both dividing and migrating cells (Mitchison 1992). Further study, in this aspect, can be done where the analysis of these NG2 positive cells, by time lapse differential interference contrast imaging, show that they are, in fact, in the process of migrating on the ECM coated surfaces. The observation of the lamellipodia is strikingly evident on the leading edges of these migrating cells, while numerous filopodia could be

present on trailing edges. This can be further investigated to determine the location of NG2/CSPG4 on either the leading or the trailing edges.

It is possible that increased cell spreading in B3 (NG2 -ve) chondrosarcoma cells may trigger other signalling pathways that lead to cytoskeleton reorganisation and increased cell spreading. This may be different from what is known about NG2/CSPG4 mediated signalling transduction pathways in studies shown before in the field of cell spreading and would be an interesting field to investigate. It is interesting to speculate that extracellular matrix ligands for NG2/CSPG4 may trigger different types of cytoskeleton rearrangements, depending on which portion of the NG2/CSPG4 ectodomain serves as the site of interaction. Clearly, it will be important for us to determine whether interaction of NG2 with physiological ligands such as type VI collagen results in responses similar to other ECM ligands. There are many possible mechanisms that could explain the possible effects of the interaction of NG2 with actin cytoskeleton or other ECM ligands. The formation of lamellipodia or filopodia occurs with the engagement of NG2 to the ECM substratum; suggest the involvement of small GTPases (rac and cdc42). However, other adaptor proteins will be required to link NG2 with these signalling pathways. MUPP, a cytoplasmic scaffolding protein, is one of these candidates that bind to the PDZ binding motif of NG2. Other adaptor proteins, such as serotonin and APC tumour suppressor protein are critical for the localisation of NG2 to the sub-cellular compartments. Expression of NG2 greatly enhances the formation of retraction fibres and the ability of cells to assume the polarised motility characteristic of motile cells.

In U251/NG2.51 cells, it has been proposed that the same complex of NG2, Cdc-42(GTP Rho family member) and p130cas, which is involved in cell adhesion, is also involved in

NG2 mediating cell spreading. This study confirmed the role of p130cas in this process and demonstrated further that NG2 is involved indirectly in this process. Growing U251/NG2.51 cells on surfaces coated with the (N143) NG2 monoclonal antibody (recognises the extracellular portion of NG2 near the trans membrane) resulted in increased expression of Cdc-42 and the formation of the lamellopodia, which indicates the involvement of GTP Rho family in this process and the connection between NG2 and this family in the mediating cell spreading process (Majumdar et al. 2003).

6.6.1.3 NG2/CSPG4 and cell migration

Regarding the migration process, in this study, we found that JJ012 (NG2+ve) cells migrate more than B3 (NG2-ve) cells, which highlights the importance of NG2 in human chondrosarcoma. The role of NG2 in cell migration has been thoroughly discussed. It has been found that the Oli-neu cell line express NG2 on their cell surface and are extremely motile (Niehaus et al. 1999). Primary oligodendrocyte cultures, Oli-neu and an immortalised Schwann cell clone showed that the polyclonal antibodies generated against NG2 inhibited cell migration (Niehaus et al. 1999; Schneider et al. 2001). Furthermore, the endothelial cell motility was enhanced by the exposure to NG2. This effect was due to the interaction of NG2 with the galectin-3 and $\alpha 3\beta 1$ complex on the endothelial cell surfaces, which leads not only to increase cell motility, but the endothelial tube formation invitro and the blood vessel development invivo (Fukushi et al. 2004).

In addition, human U251 astrocytoma cells transfected with the wild-type were more motile than those expressing ‘chimeric proteins’ where either the cytoplasmic or the transmembrane portion of NG2 had been replaced. From this study, it seems that the proximal-membrane

segment of NG2 is necessary for actin cytoskeleton reorganisation and cell motility (Fang et al. 1999).

Integrin family members can integrate with NG2 in mediating this process. There are wide varieties of integrins, which integrate with NG2/CSPG4 in the process of cell migration as well as spreading such as $\alpha 2\beta 1$, $\alpha v\beta 1$, $\alpha 4\beta 1$ and $\alpha 3\beta 1$. This is also cell-dependent, which could explain the diversity of integrin binding. Milner and his colleagues in 1996 showed that the oligodendrocytes precursors express $\alpha v\beta 1$, where the down regulation of integrin is correlated with the loss of migratory potential (Milner et al. 1996). In addition, when melanoma cells were incubated with anti-integrin antibodies, there was an inhibition of cell migration (Iida et al 1998). It has also been shown that the chondroitin sulphate glycosaminoglycan chain is required for the $\alpha v\beta 1$ binding to NG2/CSPG4 (Iida et al. 1998). This Integrin and NG2 binding trigger a cascade of intracellular signalling pathways and phosphorylation of intracellular and cytoskeletal molecules. In this study we have not investigated the mechanism behind increased cell migration in JJ012 (NG2+ve) cells. Exploring the mechanism behind NG2 increased cell migration and whether NG2 is involved directly or indirectly in cell migration of chondrocytes would be of interest.

Phosphorylation of NG2 plays an important role in cell motility. It has been shown that the phosphorylation of NG2/CSPG4 at the threonine 2256 caused the redistribution of NG2 on the cell surface of astrocytomas and a significant increase in the cell motility (Makngiasra et al 2004). The addition of PMA (phorbol myristate-acetate) (PKC- α stimulator), results in co-localisation of NG2 with both $\alpha 3\beta 1$ and ezrin-radixin-moesin in lamellopodia. When cells were treated with a selective inhibitor of PKC- α , the phosphorylation of NG2 was reduced significantly at the threonine 2256 as well as cell motility being reduced.

5.6.1.4 NG2/CSPG4 and cell proliferation

The role of NG2/CSPG4 in cell proliferation was approached in this study. This was studied in detail in both normal and diseased conditions. The idea of involvement of NG2 in cell proliferation was supported by the fact that NG2/CSPG4 is capable of interacting with high affinity to the growth factors: FGF-2(bFGF) and PDGF-AA through its core protein part rather than the chondroitin sulphate chain (Goretzki et al. 1999). There are at least 2 binding sites for PDGF-AA and FGF-2 within the ectodomain part of NG2/CSPG4. NG2/CSPG4 appears to help these growth factors, by sequestering them at the cell surface and presenting them to their respective receptors (Stallcup 2002).

An initial study carried out on O2A oligodendrocyte progenitor cells showed that treatment of these cells with NG2 blocking antibody *in vitro* inhibited the proliferation of these cells and reduced the expression of PDGF alpha- receptors (Nishiyama et al. 1996). Both NG2 and PDGF-alpha receptors are co-ordinately regulated. Furthermore, this study highlighted the importance of the co-expression of the NG2 proteoglycans and PDGF alpha-receptors on the surface of O2A progenitor cells as this determined the ability of these cells to respond effectively to the mitogenic stimulus of PDGF.

This was further supported by another work, showing that NG2-null progenitor cells (A2B5+O4-) derived from NG2 knockout mice were unable to maintain their proliferative status in response to bFGF and PDGF-AA, in comparison to the wild type (A2B5+O4-) cells (Stallcup 2002). Over a course of several days, NG2-null progenitors could not maintain their undifferentiated state, did not respond to growth factors and progressed to A2B5-O4+ phenotype characteristic of initial differentiation along the oligodendrocyte pathway.

In aortic smooth muscle cells, NG2 null cells were unable to proliferate or migrate normally in response to PDGF-AA, due to poor activation of PDGFR- α , while wild type cells exhibited a very good proliferation and migration abilities in response to this factor. However, both cell types maintained their mitogenic and proliferative properties in response to PDGF-BB (Grako et al. 1999). There was no autophosphorylation of PDGF- α receptors in treated null cells which highlights that a defective signal transduction happened at the PDGF- α receptors after NG2 loss from these cells. It also indicates that the activation of the extracellular signal-regulated kinase (ERK) in response to PDGF-AA and PDGF-BB occurs at different levels and seems that NG2 loss affects the mitogenic response to PDGF-AA rather than BB one.

There is unpublished data from Dr. Roberto Perris's laboratory and mentioned in Stallcup and Huang's review paper in 2008, that the lack of NG2 from the smooth muscle cells makes them less responsive to FGF-2, resulting in reduced activation of FGF-2 receptor and consequently low proliferative response. This was more or less similar to the idea obtained by Ozerdem and Stallcup in their published paper in 2004, where they showed that cells lacking NG2 failed to sequester FGF-2 growth factor, resulting in reduced activation of FGF-2 receptors as well as a reduction in the FGF-2 mitogenic activity. Pathological corneal and retinal angiogenesis in wild type and NG2-null mice were compared in their response to FGF-2 factor. There were twice as many ectopic vessel formations in wild-type as in NG2 null retinas, reduction in the proliferation of both pericytes and the endothelial cells and decrease in the pericytes: endothelial cells ratio. In a similar work using an invivo model of corneal angiogenesis, there was a reduced angiogenesis in FGF-2 containing corneal pellets lacking NG2 by four-fold rather than the wild-type (Ozerdem and Stallcup 2004). NG2/CSPG4 antibody significantly inhibited the angiogenesis in wild-type mice. All this

work highlights the importance of NG2/CSPG4 as an anti-angiogenic therapy in cancer treatment.

There is an important discussion about the role of phosphorylated NG2 in regulating cell proliferation. Phosphorylation of NG2 at the Thr-2314 by PKC- α caused an increase in cell proliferation. Even at the basal level without cellular stimulation NG2 transfected U251 glioma cells are more proliferative than parental U251 cells due to a higher level of phosphorylated NG2 in these cells (Makagiansar et al. 2007). It has been found that phosphorylation of NG2 at its two distinct phosphorylation sites (Thr-2256 and Thr-2314) helps to balance the two cellular functions (Motility and proliferation respectively). β 1 integrin activation appears to have an important role in this NG2 dependent cell proliferation or motility mechanisms. It appears that the nature of the stimulus determines which part of NG2 will be phosphorylated. Then, NG2/CSPG4 changes its location together with β -1-integrin to either the lamellipodia or apical microprojections, where integrins interacts with other cytoplasmic machinery and all of these can enhance either cell proliferation or cell motility. MUPP1, GRIP1, syntenin-1 and ezrin are possible candidates for such anchoring functions (Barritt et al. 2000; Stegmuller et al. 2003; Makagiansar et al. 2004; Chatterjee et al. 2008).

5.6.1.5 NG2/CSPG4 and chemoresistance

To study the role of NG2/CSPG4 in human chondrosarcoma, I assessed the treatment of JJ012 chondrosarcoma cell line with chemotherapeutic drugs by MTT assay. The first aim was to choose the best seeding density to be used for the dose response curve. It has been shown that anti-cancer therapy can be affected by the cell confluency as the effect of anti-

cancer drugs were decreased with increasing cell density (Pelletier et al. 1990). Dose response curves for chemotherapeutic drugs were performed on JJ012 cells to get the IC₅₀ dose. It was possible to calculate the IC₅₀ for doxorubicin and docetaxel. However, there was a rapid decrease in cell viability for cisplatin between (10 and 30µM) and ifosfamide (between 3 and 10mM). This could be due to either to real drug effect or it could be attributed to the drug's cytotoxicity. Therefore, this was further assessed by annexin V labelling to measure apoptosis/necrosis, because anti-cancer drugs are supposed to induce apoptosis in susceptible cells. Results confirmed that both doxorubicin and docetaxel can be used in our study, while ifosfamide and cisplatin are not good models in our study as they induced more necrosis than apoptosis in these cells.

Although MTT assay results did not show a statistically significant difference in the cellular response to doxorubicin treatment (0.3µM) between JJ012 (NG2+ve) and B3 (NG2-ve) cells, annexin V labelling results did. There was a statistically significant difference in the apoptosis percentage between JJ012 (NG2+ve) and B3 (NG2-ve) cells after 0.3µM doxorubicin treatment for 48. More interestingly, at the 72 hours time point, while B3 (NG2-ve) cells had a positive apoptosis percentage, JJ012 (NG2+ve) cells had a negative apoptosis percentage with no significant difference observed. For Docetaxel treatment (10nm), both MTT results annexin V staining showed no significant difference in the cellular response between JJ012 (NG2+ve) and B3 (NG2-ve) cells.

More interest has arisen recently in the role of NG2/CSPG4 in regulating cell death signalling (Chekenya et al. 2008). The function of NG2/CSPG4 in human chondrosarcoma and chondrocytes in general is as yet unclear. The apoptotic death of articular chondrocytes has been implicated in the pathogenesis of osteoarthritis and it is possible that NG2 might be involved (Midwood and Salter 2001; Kim and Blanco 2007). The expression of NG2/CSPG4

in human chondrosarcoma raises the possibility that NG2/CSPG4 may have an important role in this tumour. Chondrosarcoma is regarded as a chemo- and radio-resistant tumour. NG2/CSPG4 is over-expressed by several tumour types that fail to respond to conventional chemotherapy, including Glioblastomas, melanomas and some leukaemia (Schrappe et al. 1991; Behm et al. 1996; Li et al. 2003). Uveal melanomas are known as chemo-resistant tumour, but are more responsive to radio- and immunotherapy ((Flaherty et al. 1998). In uveal human melanoma paraffin sections, NG2/CSPG4 strong immunoreactivity was detected using monoclonal antibody 9. 2. 27, the latter was used as a molecular targeting in targeted alpha therapy for melanoma, suggesting that this antibody can be used as an effective treatment for human uveal melanomas (Li et al. 2003).

Gliomas are another chemo and radio-resistant tumour, which fail to respond to conventional chemotherapy regimens (Holland 2000; Giese et al. 2003). In these tumours, it has been found that NG2/CSPG4 is not only expressed by the glioma cells and by oligodendrocytes progenitors, but also by the pericytes associated with the tumour vasculature. In addition, NG2/CSPG4 expression was also confined to tumour areas with high cellular proliferations, suggested that NG2/CSPG4 can have an important role in the treatment of these tumours (Chekenya et al. 2002). All these studies highlighted the importance of NG2/CSPG4 in the tumours in general and chemo-resistant tumours specifically, and raise the possibility that NG2/CSPG4 may have an important role in human chondrosarcoma, as this tumour is regarded as a chemo resistant tumour.

We have not studied the mechanism responsible for the doxorubicin chemoresistance in JJ012 cells. The effect of doxorubicin treatment was studied before in JJ012 human

chondrosarcoma cell line, using different doses of doxorubicin (0.1 and 1 μ M) (Kim et al. 2007). In the latter study, it has been shown that the anti-apoptotic proteins are responsible for the apoptosis resistance in human chondrosarcoma, in addition to P-glycoprotein. Although JJ012 and doxorubicin treatment was used in the latter study, the experimental approach was different from the one used in this thesis. The role of NG2/CSPG4 mediated chemoresistance in human glioblastoma multiforme cell line was studied *in vitro* and was confirmed by human GBM spheroids from patients. The data from both experiments showed the same effect. that the activation of integrin dependent PI3K/Akt signalling was behind this response (Chekenya et al. 2008). In glioblastomas, NG2-transfected U251 glioma cells were resistant to cytotoxic drugs (Doxorubicin, Vincristine and Etoposide) and TNF- α through increased activity of $\alpha 3 \beta 1$ integrin/ PI3K signalling and their downstream targets, which all promote cell survival (Joy et al. 2003; Downward 2004; Chekenya et al. 2008). siRNA mediated NG2/CSPG4 knockdown sensitised the NG2-transfected U251 glioma cells to cytotoxic treatment, which further supports the previous data and highlights the relationship between NG2 and the apoptosis resistance. In the same study, it has been found that NG2 knockdown was effective in establishing the apoptosis sensitivity in endogenous NG2 expressing glioma lines such as U87 and A172, as well as in the A375 melanoma line, which suggests that NG2/CSPG4 may be an effective therapeutic target in several cancer subtypes (Chekenya et al. 2008). The role of NG2 dependent apoptosis mechanism in this study was further supported by *in vivo* work, whereby the presence of NG2 enhances the tumour growth, promotes the chemoresistance to cytotoxic drugs and demonstrates a strong correlation between the level of NG2 expression and the apoptosis resistance in tumour samples.

On the other hand, NG2/CSPG4 can serve as a pro-apoptotic receptor. NG2/CSPG4 induced anoikis mechanism in fibroblasts by opposing fibronectin –stimulated $\alpha 4 \beta 1$ integrin

through PKC alpha dependent mechanism (Joo et al. 2008). This is what has been shown in fibroblast by Joo and his colleagues in 2008. It is known that altered fibronectin, causes a disruption of cell–matrix interactions and led to anoikis – apoptosis, in which reduced FAK phosphorylation is the main molecule involved in this process (Tafolla et al 2005, Kapila et al 2002 and Kapila et al 1999). It is known that NG2 regulates FAK phosphorylation through PKC- α in human melanoma cells and NG2 and $\alpha 4\beta 1$ integrin acts as co-receptors mediating the spreading of melanoma cells on FN coated surfaces (Iida et al. 1998; Yang et al. 2004). In this study, altered FN caused a reduced phosphorylation of FAK, up-regulation of NG2 and down-regulation of integrin $\alpha 4$. The role of NG2/CSPG4 in this study was opposite to integrins in regulating the anoikis and NG2 and integrin $\alpha 4$ regulate FAK phosphorylation by PKC- α dependent and -independent pathways respectively.

So, it seems to be that NG2/CSPG4 is involved in both anoikis and apoptosis resistant mechanisms, depending on the nature of the stimulus, cell types and perhaps other factors not yet explored. This indicates that NG2 mediated apoptosis resistance to doxorubicin treatment through integrin signalling may be one of the possible mechanisms in our study. Adhesion mediated apoptosis resistance is an emerging concept that explained why cancer cells are resistant to apoptosis. Cell-cell interaction, cell- matrix or cell- stroma interactions are also other influential factors in this aspect (Westhoff and Fulda 2009).

There were different cellular responses to different chemotherapeutic drugs in this study. No apoptosis was induced in JJ012 cells by using both Ifosfamide and Cisplatin. Although apoptosis was induced in JJJ012 cells by using doxorubicin and docetaxel, NG2 was able to interfere with the action of doxorubicin only. Anti-cancer drugs are originally classified either by their origin or their target molecules. It is well known that anti-cancer drugs induce

apoptosis in cancer cells (Fisher 1994; Hickman et al. 1994; Wong 2011; Mansilla et al. 2012). Cisplatin is an alkylating agent, which reacts with DNA and cellular proteins. The primary mode of action is cross linking DNA and inhibiting replication of DNA and the transcription of RNA (Jamieson and Boddy 2011). Doxorubicin action is mainly through intercalating between the two DNA stands, preventing the topoisomerase II enzymatic activity and leads to DNA damage. The mechanism of action of Docetaxel is different. It mainly interferes with the cell division, preventing the microtubules assembly, and initiate apoptosis. Apoptosis is further encouraged by blocking of apoptosis-blocking Bcl-2 oncoprotein. Ifosfamide is a nitrogen mustard alkalayting agent that stops tumour growth by cross linking guanine nucleobases in DNA double-helix strands, directly attacking DNA. This makes the strands unable to uncoil and separate. As this is necessary in DNA replication, the cells can no longer divide. These drugs act non-specifically. Therefore, it seems to be that the drugs used in this study have different mechanisms of actions. There are different mechanisms of how cancer drugs exert their effects inside cells such as efflux pumps, drug uptake and metabolism, drug targets inside cells, compartmentalisation of the drug inside cells, the effect of the drug on cell cycle checkpoints and the balance between repair of damaged DNA and the induction of apoptosis. NG2 may have a selective effect in the response to one chemotherapeutic drug and not to the other one, by interfering with one of these mechanisms mentioned. Micro-arrays study could help us to understand the possible affected genes between cells treated with different chemotherapeutic drugs. Studying the mechanisms of cancer drug resistance, possible molecular targets affected, and investigating any difference in the expression may give us an explanation for that. This selective effect of NG2 in mediating protection from apoptosis induced by anti-cancer therapy is not clear in our study and further work is necessary to get the possible answer. This can also explained by

further investigating the signalling pathway associated with NG2 role in doxorubicin mediated chemo resistance in JJ012.

5.6.2 The effect of NG2/CSPG4 on the markers involved in OA process

5.6.2.1 NG2/CSPG4 and OA specific markers

Osteoarthritis (OA) is a disease characterised by progressive destruction of the joints, and although disease initiation may be multi-factorial, osteoarthritis appears to be a result of uncontrolled proteolytic extracellular matrix destruction. Aggrecan is the major component of the extracellular matrix of the cartilage. In the early events of OA where cartilage remodelling process occurs, aggrecanases are the main proteinases responsible for aggrecan cleavage. After that, MMPs actions are noticed in the development of OA process and continue with the destruction of collagen (Nagase and Kashiwagi 2003). Therefore, aggrecanase mediated cartilage destruction is considered a characteristic feature of cartilage degradation during inflammatory joint diseases like OA. ADAMTS-4 and 5, together with ADAMTS-8, 9 and 1 are regarded as the main aggrecanases responsible for cartilage proteoglycan destruction in arthritis in general (Kuno et al. 2000; Tortorella et al. 2000; Tortorella et al. 2001; Kuno et al. 2004).

In the cartilage, two different aggrecanases, i.e., aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5) have been isolated. A tight regulation of these aggrecanases activity is very important for maintaining a good balance between aggrecan anabolism and catabolism. In the normal human body, endogenous inhibitors like, tissue inhibitor of matrix metalloproteinase (TIMP-3) of the aggrecanases controls the mechanism for aggrecan catabolism. However, in diseases such as OA, the balance between TIMP-3 and ADAMTS-4

synthesis is disturbed, favouring the catabolism state. This disturbance could be related to two factors: *de novo* synthesis of ADAMTS-4 (Curtis et al. 2002; Malfait et al. 2002) and/or post-translational activation of ADAMTS-4 and ADAMTS-5.

In this study, a reduced expression of ADMATS-4 and complete loss of ADAMTS-5 were found in NG2 knockdown cells. In the literature, the link between NG2 and ADMATS-4 and 5 is not yet known. This could be an interesting point to investigate, as mentioned earlier, ADMATS-4 and 5 play an important role in the pathogenesis of OA. Therefore, the differential effect of NG2 on these aggrecanases may indicate the importance of this molecule in OA process.

Several studies highlighted the differences between ADAMTS-4 and 5. ADAMTS-5 and 4 are two shorter members of the zinc-dependent ADAMTs enzyme family (Tang 2001) and has two thrombospondin (TS) motifs and only one TS motif respectively. ADAMTS-4 and ADAMTS-5 are two of the most active enzymes in aggrecan cleavage, with ADAMTS-4 is mainly active during cartilage degeneration, and is up regulated in degenerative cartilage (Tortorella et al. 2002; Song et al. 2007). However, ADAMTS-5 has been shown to be active in both normal and degenerated cartilage (Kevorkian et al. 2004). Even with the degeneration of both ADAMTS-4 and 5 knockout mice, it is not yet known which of the ADAMTs family members is more important in the development of cartilage degenerative diseases. Although both ADAMTS-4 and 5 knockout mice are normal and have no gross abnormalities (Glasson et al. 2004; Glasson et al. 2005), it was found that ADMATS-5 may play a more important role in OA development than ADMATS-4.

Meniscal destabilisation experiments were performed in both ADAMTS-4 and 5 knockout mice, showing that deletion of the ADMATS-4 gene cannot protect OA progression. However, deletion of the ADAMTS-5 gene alone decelerated cartilage degradation (Lohmander et al. 2003; Stanton et al. 2005). To define the peptidases that are truly active in OA *in vivo*, the detection of specific cleavage products in the cartilage, synovial fluid, serum, or urine was performed. Measurement of transcript abundance by quantitative PCR and/or peptidase abundance by immunoassay can generate correlative data, but they are not precise enough to provide the definitive proof obtained with highly specific neo-epitope antibodies. Therefore, only a limited number of peptidases can be directly implicated in human OA cartilage pathology. These include one that cleaves in the C-telopeptide region of collagen II (Lohmander et al. 2003); probably MMP13, which cleaves collagen II at (Poole et al. 2004; Charni et al. 2005) Cathepsin K, which degrades collagen II (Dejica et al. 2008) and an ADAMTs-aggrecanase, which cleaves aggrecan (Poole et al. 2004; Charni et al. 2005; Dejica et al. 2008). Further studies on how ADAMTS-5 deletion protects cartilage demonstrated that this activity is not only due to elimination of aggrecanase activity from the cartilage (Li et al.). However, a photographic, histologic, and biochemical examination of the “protected” joints in ADAMTS-5 knockout mice provided evidence that the protection was, mainly, due to an elimination of fibrous overgrowth from the peri-articular tissues and deposition of newly synthesised aggrecan in the cartilage (Li et al.). This result suggests that ADAMTS-5 activity is pro-fibrogenic in the joint responses to injury, with the deletion of the ADAMTS-5 gene shifts cells to a chondrogenic phenotype. The possible explanation for these effects is that ADAMTS-5 knockout on collagenous tissues is that, in the presence of the enzyme, TGF- β 1-mediated signalling is primarily through Smad2/3, leading to increased expression of fibrogenic genes such as type I and type III collagen. Conversely, in the absence of the enzyme and the presence of accumulated pericellular aggrecan, TGF β 1 mediated signalling is

primarily through the Smad1/5/8 pathway. Activation of this pathway, which is also activated by BMP7 signalling, activates expression of chondrogenic genes such as aggrecan (Plaas et al. 2011). The precise mechanism behind this is not known, but analysis of ADAMTS-5/CD44 double knockout mice shows that it is dependent on the presence of the hyaluronan receptor, CD44. These data concluded that ADAMTS-5 specifically degrades pericellular aggrecan in OA and that other aggrecanases, such as ADAMTS-4, are responsible for degradation of the bulk of the tissue aggrecan, which resides in the intercellular matrix.

The central event of OA process is the progressive loss of articular cartilage. Studies carried out on human clinical and animal tissues indicated that MMP13 plays a dominant role in the progression of cartilage degeneration. It possesses a more restricted expression pattern in connective tissue in comparison to other MMPs (Vincenti and Brinckerhoff 2002). MMP13 is a collagenase which targets specifically collagen II, which is one of the abundant proteins in articular cartilage. It also degrades aggrecan, types IV and IX collagen, gelatin, osteonectin, and perlecan in cartilage. MMP13 has a much higher catalytic velocity rate than other MMPs for Collagen II and gelatin. This suggests that MMP13 is most potent peptidolytic enzyme among collagenase (Knauper et al. 1996; Reboul et al. 1996).

A study revealed that patients with articular cartilage destruction have high MMP13 expression, so increased MMP13 may be a cause of cartilage degradation (Roach et al. 2005). Furthermore, MMP13- deficient mice has no gross phenotypic abnormalities, however only alteration in growth plate architecture was noted (Inada et al. 2004; Stickens et al. 2004). MMP13-overexpressing transgenic mice developed spontaneous articular cartilage destruction, where there was excessive cleavage of collagen II and loss of aggrecan (Neuhold et al. 2001). Consistent with the above findings; results from chondrocyte-specific MMP13-

conditional knockout (MMP13 cKO) mice showed decelerated OA progression following meniscal-ligamentous injury (MLI). It has been found that the inhibition of MMP13 could serve as a potential therapeutic option for OA treatment by the use of CL82198, a MMP13 inhibitor into WT mice after MLI surgery. The injection of CL82198 decelerated MLI-induced OA progression. These findings implicate MMP13 as a critical player in the progression of OA and suggest that it could serve as a molecular target for OA therapies.

In addition, it has been found that there is a good correlation between the degree of malignancy in chondrosarcoma and the expression of matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase (ADAM) with thrombospondin motifs (ADAMTSs) and tissue inhibitor of metalloproteinases (TIMPs). The study was carried out on 28 chondrosarcoma cases. There was a significant positive correlation ($p < 0.05$) present between immunolabelling and histological grades for all proteins tested (MMP 7, ADMATS 4 and 5) and TIMP 1, 2, 3 with strong correlations ($p < 0.01$) for MMPs 2, 3 and 13, both ADAMTSs and all 3 TIMPs. However, no correlation was found between the prognosis and immunostaining scores. So, the conclusion was, these proteins can be used to assess the degree of malignancy in human chondrosarcoma (Sugita et al. 2004)

In the literature, the association between NG2 with MMP3 has not yet been studied. Results of this study showed an increased expression of MMP13 and reduced and a complete loss of ADAMTS-4 and 5 were found in NG2 knockdown cells. From all of these, it seems that NG2 has a differential effect on the enzymes responsible for cartilage destruction in OA process, which results in selective degradation of aggrecan and collagen II, the main constituents of ECM of the cartilage. More investigation is required in this field, using the quantitative qPCR

to accurately estimate the amount of these two products on NG2 -ve cells may be the answer behind this.

In OA process, there are morphological changes observed include cartilage erosion, which are accompanied by a variable degree of synovial inflammation. A complex network of biochemical factors, including proteolytic enzymes, lead to a breakdown of the cartilage macromolecules. Cytokines such as IL-1 and TNF-alpha, which are produced by activated synoviocytes, mononuclear cells or by articular cartilage itself, all significantly up-regulate metalloproteinase (MMP) gene expression. It has been found that, in OA, dysregulated cytokine activities disrupt the balance between anabolism and catabolism (Lin et al. 2009; Yang et al. 2010).

5.6.2.2 NG2/CSPG4 and pro-and anti-inflammatory cytokines

In vitro and in vivo studies emphasised the role of the pro-inflammatory cytokines, particularly interleukin (IL) 1 and tumour necrosis factor (TNF), in cartilage pathology in rheumatoid arthritis and OA (Vincenti and Brinckerhoff 2002; Mak et al. 2006). The chondrocyte is the cellular target of cytokine action in cartilage (Shiomi et al. 2010). Cytokines also decrease the chondrocyte compensatory synthesis pathways required to restore the integrity of the degraded extracellular matrix (ECM).

It has been shown that IL1 β suppresses the expression of a number of genes associated with the differentiated chondrocyte phenotype, including COL2A1 as well as increasing destructive proteinases (Knauper et al. 1996; Roach et al. 2005). IL1 and TNF α can inhibit the synthesis of type II collagen by chondrocytes by suppressing gene transcription (Knauper et al. 1996; Reboul et al. 1996; Stickens et al. 2004) and stimulate the synthesis of

prostaglandin E2, which feedback regulates COL2A1 transcription in a positive manner (Knauper et al. 1996; Reboul et al. 1996; Neuhold et al. 2001) depending upon receptor utilisation (Tang 2001). In addition, there is a relative deficit in the production of natural antagonists of the IL-1 receptor (IL-1Ra), which could possibly be related to an excess production of nitric oxide in OA tissues as well as up regulation in the receptor level, All of these are shown to enhance the catabolic effect of IL-1 in this disease. All of this counter acts the effect of IL-1 and/or TNF- α up-regulation of MMP gene expression and appears to be a potential medical therapy of OA.

Therefore, it appears that IL-1 β is extremely important to cartilage destruction contributing to an increased production of matrix metalloproteinases (MMPs) and other degenerative products, while TNF- α appears to drive the inflammatory process. In this study, although there was no difference observed in the level of expression of IL-1 β between JJ012 (NG2+ve) and B3 (NG2-ve) cells, there was a reduced expression of MMP3 and increased expression of MMP13 in B3 (NG2-ve) cells. From all of these data, it is possible to conclude that NG2 might have a differential effect on different signal pathways involved in OA pathogenesis, and IL-1b independent reduced expression of MMP3 accompanied by increase expression of MMP13 may possibly occur in our system.

Certain cytokines such as IL-4, IL-10, and IL-13 have their anti-inflammatory properties, which appear to depend greatly on the target cell. *In vitro* study showed that Interleukin-4 (IL-4) has been shown to suppress the synthesis of both TNF- α and IL-1 β in the same manner as low-dose dexamethasone in OA process. Naturally occurring anti-inflammatory cytokines such as IL-10 inhibit the synthesis of IL-1 and TNF- α and can be potential therapeutic targets in OA. Similarly, IL-13 significantly inhibits lipopolysaccharide (LPS)-induced TNF- α

production by mononuclear cells from peripheral blood, but not in cells from inflamed synovial fluid. Its important biological activities include: inhibition of the production of a wide range of pro-inflammatory cytokines in monocytes/macrophages, B cells, natural killer cells and endothelial cells, while increasing IL-1Ra production. In OA synovial membranes treated with LPS, IL-13 inhibited the synthesis of IL-1 β , TNF- α and stromelysin, while increasing IL-1Ra production (Kapoor et al. 2010). IL-2RG is considered as an important signalling component of many other interleukin receptors, including those of interleukin (Verzija et al. 2000; Rousseau et al. 2004; Hunter et al. 2005), and is, thus, referred to as the common gamma chain. Deficiency of this receptor causes severe combined immunodeficiency disorder. However, its relation to OA has not been approached yet.

The data in this study showed that there was a reduced expression of IL-10, no difference in the expression of IL-4 and IL-13 alpha 1 and 2 receptors, and increased expression of IL-2RG. Although it appears that NG2 possess a differential effect on the cytokines involved in OA process, further studies are required to assess the effect of NG2/CSPG4 knockdown on the cytokine and its matching cell surface receptor to further support this data.

It is well known that in OA process, chondrocytes are subjected to variety of stressors, such as oxidative, osmotic, and biomechanical stress (Lee et al. 2002; Pfander and Gelse 2007; Wuerz et al. 2007). When chondrocytes are subjected to physiological and pharmacological agents, they undergo ER stress in-vitro (Yang et al. 2005); therefore, mechanisms must exist to maintain homeostasis in spite of these stressors.

5.6.2.3NG2/CSPG4 and ER stress markers

In this study, I tested the effect of NG2 loss on different ER stress markers: Hsp70, Grp78, Bag-1 and PDI. Grp78 (known as immunoglobulin-binding protein (BiP)) and the heat shock protein-70 (hsp70) family have the following functions within the ER: 1) Translocation of newly synthesised proteins across the ER membrane, 2) Maintaining proteins in a form that prevents improper aggregation, 3) Allowing proper protein folding and oligerimization, 4) Targeting of misfolded proteins for proteasomal degradation (Lee et al. 2002; Hendershot 2004; Li and Lee 2006). Therefore, Grp78 is a target gene of the unfolded protein response (UPR) signaling pathway in the ER and it is up regulated in ER stress (Lee 2001; Banhegyi et al. 2007).

Bag-1 has recently been shown to be a downstream regulator of ER stress in chondrocytes. One study showed that, in-vitro, there was a down regulation of Bag-1 in exogenously induced ER- stress in mammalian chondrocytes. Moreover, over expression of bag-1 protected chondrocytes from ER stress-induced down regulation of collagen type II and apoptosis (Yang et al. 2007). So, the hypothesis from all of these data was, if chondrocytes do, in fact, undergo ER stress during the pathogenesis of OA, an up regulation of Grp78 might happen, which may or may not be associated with a down regulation of bag-1.

This is followed by another paper done in 2009 by Nugent and his colleagues. It showed that there was an up regulation of Grp78 in advanced OA, and variable expression in minimal OA. Non-OA cartilage was, however, Grp78 negative. The downstream regulator bag-1 was also up-regulated in OA compared with normal cartilage. These data were supported by investigating the level of collagen VI and NG2 (to assess the health and the turnover of the cartilage in health and disease conditions). Collagen VI had a more widespread distribution in

OA cartilage, along with increased intracellular labelling intensity. NG2/CSPG4 was down regulated in advanced OA compared with its patient matched minimally involved cartilage sample. The conclusion was that, chondrocytes undergo ER stress in OA, with up regulation of both Grp78 and Bag-1. This is supported by other findings (already known) of ECM changes in OA process (Nugent et al. 2009). Protein disulfide isomerase or PDI is an enzyme in the endoplasmic reticulum that catalyses the formation and breakage of disulfide bonds between cysteine residues within proteins as they fold (from Wikipedia).

In this study, there was an NG2 differential response on ER stress marker. Although there was no effect on the level of expression in Grp78, Hsp70 and Bag-1, an increase in the level of expression of PDI was noted in NG2 knockdown cells. Apart from the literature mentioned above still very little known about the role of NG2 and ER stress marker. For OA process, all studies support the idea that chondrocytes undergo ER stress, supported by an increase in the level of expression of ER stress marker. However, not all ER stress markers are being investigated, like in the case of PDI. An increase in the level of PDI with NG2 loss may have an important role in OA process by protecting cells from having more severe OA changes. Therefore, further studies will be required to understand the differential effects of NG2 on ER stress markers by exploring the signalling pathways behind this.

5.6.2.4 NG2/CSPG4 and its extracellular ligands

It was interesting to investigate the effect of NG2 knockdown on its extracellular ligands in JJ012 chondrosarcoma cells. It is well known that collagen VI appears to be a cell surface ligand for NG2 and NG2 is associated with fibroblast growth factor/ fibroblast growth factor receptor (FGF/FGFR) (Stallcup and Huang 2008). Despite that, results showed that there was no effect of NG2 knockdown on the expression level of collagen VI or FGF-2, however,

there was a complete loss of the expression of PDGFA, with increased expression of PDGFB in NG2 knocked down cells being observed.

The PDGF family of growth factors is of four different polypeptide chains, encoded by four different genes, with the classical PDGF chains of PDGF-A and PDGF. The four PDGF chains known are: PDGF-AA, PDGFAB, PDGF-BB, PDGF-CC and PDGF-DD. The dimeric ligands (PDGF-AA, -AB, and -BB) exert their biological effects by binding to two monomeric units of the receptors, PDGFR- α or - β , each with distinct binding properties (Chen et al. 2012)

PDGFR- α show a high affinity for both the PDGF-A and -B chains, while PDGFR- β preferentially binds the B chain only. Receptor dimerisation is closely related to the activation of the intrinsic protein tyrosine kinase (Chen et al. 2012). Functional differences between PDGFR- α and - β have been reported in actin organization of porcine aortic endothelial cells (Eriksson et al. 1992), in the chemotaxis on human foreskin fibroblasts (Vassbotn et al. 1992), and in transformation of NIW3T3 fibroblasts (Heidaran et al. 1993). It is well known that PDGFR- α and - β are both equally capable of triggering DNA synthesis in any type of cell (Eriksson et al. 1992; Heidaran et al. 1993; Chen et al. 2012), however, the difference in the mitogenic sensitivity to PDGF isoforms is attributed to the differences in expression levels of the two receptor isoforms which vary depending on the cell type (Chen et al. 2012). Earlier studies showed that PDGF is one of the major mitogen in serum and is responsible for proliferation of certain cell types, including VSMC (Ross et al. 1986; Seifert et al. 1989). One of these demonstrates the differential cellular response to PDGF isoforms in cultured rat VSMC and it was found that the PDGF-BB isoform was a potent mitogen. It was also found that PDGFAA was not and this was mainly attributed to the absence of expression

of PDGFR- α (Inui et al. 1993; Kondo et al. 1993). NG2/CSPG4 facilitates the activity of these growth factors, by sequestering them at the cell surface and presenting them to their respective receptors (Stallcup 2002).

The effect of NG2 loss on PDGFs is differential. Although there was a complete loss of the expression of PDGFA, with increased expression of PDGFB in NG2 knocked down cells, further studies in this field are required to investigate the effect of NG2 loss on PDGF receptors. Several studies supported the data, where it has been shown that there is a direct link between NG2 and PDGF-AA, and the disturbances of this has its functional implication on the cellular processes.

The action of specific growth factors, such as PDGF, bFGF, EGF, and IGF-1 on the behaviour of vascular smooth muscle cells is apparent during development and in a variety of pathological situations, which has been discussed previously in many studies (Lindner et al. 1990; Ferns et al. 1991; Lindner and Reidy 1991; Yamamoto and Yamamoto 1994).

These growth factors involve smooth muscle cells proliferation, migration, and differentiation in response to changes in the local environment. The membrane spanning chondroitin sulfate proteoglycan NG2 plays an important role in the ability of aortic smooth muscle cells to respond to platelet derived growth factor (PDGF). Interestingly, the aortic smooth muscle cells did not undergo mitosis and migration in response to PDGF-AA was inhibited by antibodies against NG2. However, anti-NG2 antibodies did not affect mitosis or migration induced by PDGF-BB (Grako and Stallcup 1995). It appears that, from these results, signaling through the PDGF - α receptor is specifically disrupted in some fashion by the anti-NG2 antibodies, since PDGF-AA activates the PDGF α -receptor, but not the PDGF - β

receptor (Seifert et al. 1989). In addition, several proteoglycans, including syndecan, betaglycan, and decorin, are thought to be involved in modulating or facilitating cellular responses to growth factors (Kaname and Ruoslahti 1996; Steinfeld et al. 1996).

Another study in aortic smooth muscle cells investigated the role of NG2 and PDGF-AA. NG2 deficient cells were unable to proliferate or migrate in response to PDGF-AA, while wild type cells exhibited a very good proliferation and migration ability in response to this factor. However, both cell types maintained their mitogenic and proliferative properties in response to PDGF-BB (Grako et al. 1999). This occurs mainly due to poor activation of PDGF receptor- α and no autophosphorylation of PDGF receptor- α in treated null-cells, which are regarded as an early steps in the signalling pathway (MAP kinase cascade leading to phosphorylation of the extracellular signal-regulated kinase (ERK) in response to PDGF-AA). Therefore, this highlights that a defective signal transduction occurred at the PDGF- α receptors after NG2 loss from these cells. It also demonstrated that the activation of the extracellular signal-regulated kinase (ERK), in response to PDGF-AA and PDGF-BB, occurs at different levels and seems that NG2 loss affects the mitogenic response to PDGF-AA rather than BB factor. These observations therefore established an important role of NG2/CSPG4 in potentiating the activation of the PDGF α -receptor by PDGF-AA.

In the central nervous system, the same findings are obtained in studies of oligodendrocyte progenitor cells. During central nervous system development, NG2 and PDGF- α receptor are co-expressed by immature oligodendrocyte progenitors and to be co-ordinately down regulated as the progenitors differentiated into oligodendrocytes (Nishiyama et al. 1996). In *in vitro*, NG2 and PDGF α -receptor were present and co-localised to the same cell surface microdomains of oligodendrocyte progenitor cells and were co-ordinately up- or down

regulated respectively by exposure to or withdrawal from bFGF. This supported the existence of an association between the two molecules. Similar to the finding in aortic smooth muscle cells, the ability of oligodendrocyte progenitors to proliferate in response to PDGF was blocked by antibodies against NG2 (Nishiyama et al. 1996). Furthermore, NG2-null progenitor cells (A2B5+O4⁻) derived from NG2 knockout mice, were unable to maintain their proliferative ability in response to bFGF and PDGF-AA, while wild type (A2B5+O4⁻) cells did (Stallcup 2002). Over a course of several days, NG2-null progenitors did not maintain their undifferentiated state, did not respond to growth factors and did not progress to A2B5⁻O4⁺ phenotype, characteristic of initial differentiation along the oligodendrocyte pathway.

CHAPTER SIX

Conclusions

6.1 Conclusions

Data generated by this thesis showed that NG2/CSPG4 is expressed by JJ012 grade II human chondrosarcoma cell line, human OA chondrocytes and by C₂₀A₄ chondrocyte cell line. The expression was confirmed by RT-PCR, western blotting, indirect immunofluorescence and flow cytometry. Strong immunoreactivity of NG2/CSPG4 in human chondrosarcoma samples (i.e. *in vivo*) was detected. This is also confirmed by RT-PCR.

In vitro, different molecular weight forms of NG2/CSPG4 expressed by JJ012 grade II human chondrosarcoma cell line, human OA chondrocytes and by C₂₀A₄ chondrocyte cell line were detected. Biochemical analysis of NG2/CSPG4 expression showed that it is expressed as chondroitin sulphate-free molecule in both JJ012 and C₂₀A₄. Using two different anti-NG2/CSPG4 antibodies, human OA chondrocytes express multiple molecular weight forms differentially recognised with and without chondroitinase ABC pre-treatment. These included: a chondroitin sulphated form of 400kD, a core protein of 270kD, with further as yet unidentified bands at 238, 117 and 55kD. Indirect immunofluorescence provided evidence that the expression of NG2/CSPG4 in JJ012 cells was predominantly membrane associated whilst in OA chondrocytes and C₂₀A₄ cells, an additional predominant punctate cytoplasmic distribution was evident. In human OA chondrocytes, NG2/CSPG4 co-localised with the Golgi complex and ER. Furthermore, immunoprecipitation and mass spectrometry data showed that NG2/CSPG4 associated with type VI collagen and thrombopoietin. This may suggest different roles of NG2/CSPG4 in different diseased conditions and highlight the

importance of exploring more about the function of this receptor in osteoarthritis and chondrosarcoma.

The development of a chondrocyte cell line that has stable knockdown of NG2/CSPG4 helped to explore more about the roles of NG2/CSPG4 in human chondrocytes.

Using the stable NG2/CSPG4 knockdown model, I showed that there was no difference in cell adhesion to collagen I, II, VI and fibronectin. Using $\beta 1$ and $\alpha V\beta 3$ integrin antibodies, results showed that there was an involvement of $\beta 1$ but not $\alpha V\beta 3$ integrin in the adhesion process in human chondrosarcoma. In addition, when JJ012 cells were allowed to adhere to fibronectin coated wells and pre-treated with EGTA and EDTA, EGTA inhibited cell adhesion to fibronectin in dose-dependent manner with no significant difference observed between both JJ012 and B3 cells. EDTA reduced adhesion of B3 cells but not JJ012 to fibronectin. I concluded that, in JJ012 cells, the adhesion mechanism appears to be calcium dependent and the loss of NG2/CSPG4 renders the cells to rely on other molecules for the adhesion mechanism. NG2/CSPG4 knockdown reduced the cellular migration and proliferation (significant difference observed), increased the chondrocyte cell spreading with no difference in the background apoptosis observed at different time points used. A significant number of cells were apoptotic after Doxorubicin treatment in B3 cells. Although there was no adequate sampling for the invasion assay, preliminary data showed that there was no difference in cell invasion process between JJ012 (NG2 +ve) cells and B3 (NG2-ve) cells.

The loss of NG2/CSPG4 had differential effects on the expression of aggrecanases and MMPs, important enzymes involved in cartilage degradation in OA process. After NG2/CSPG4 loss, there was no difference in the expression of the cartilage specific markers (aggrecan, collagen II and SOX-9). In contrast, B3 cells showed a decreased expression of MMP3 and ADAMTS-4, a complete loss of ADAMTS-5 and increased expression of MMP13. The effect of NG2/CSPG4 knockdown on the pro-and anti-inflammatory cytokines involved in OA process was differential, with no effect on expression of IL-1 β , reduced expression of the anti-inflammatory cytokine IL10 in B3 (NG2-ve) cells in comparison to JJ012 (NG2+ve) cells, an increased expression of IL2RG receptor in B3 (NG2-ve) cells, with no difference in the expression level of IL13R alpha1 and 2 and IL4R between B3 (NG2-ve) cells and JJ012 (NG2+ve) cells. For ER stress markers, there was no effect of NG2/CSPG4 loss on the expression level of GRP78, HSP70 and Bag-1, while increased expression of PDI was observed in B3 (NG2-ve) cells. There was no effect on the expression level of collagen VI and FGF-2, a complete loss of the expression of PDGFA, with increased expression of PDGFB in NG2 knocked down cells was observed. A proposed model for the role of NG2/CSPG4 in human chondrocytes is shown below:

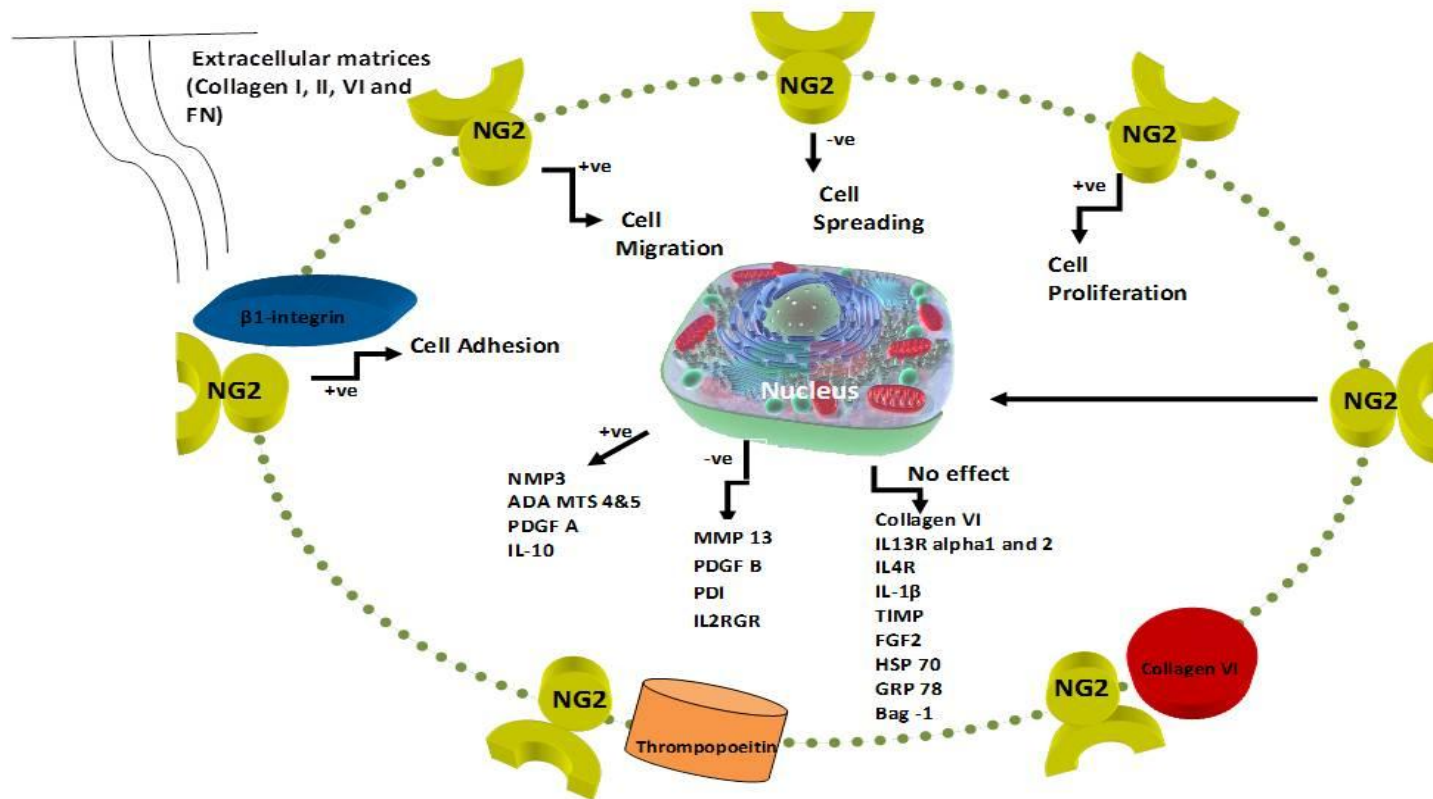


Figure 6.1: Functional roles of NG2/CSPG4 in human chondrocytes. NG2/CSPG4 promotes cell migration, proliferation and reduces cell spreading in JJ012 chondrosarcoma cells. The intracellular mechanisms behind these responses are not known yet. NG2-mediated integrin signaling is involved in cell adhesion. NG2/CSPG4 is associated with type VI collagen and thrombopoietin in human OA chondrocytes. NG2 has its differential effects (+ve (increased), -ve (decreased) and no effect) on different cellular genes, including those involved in OA process.

Taken together, all of these data indicate that NG2/CSPG4 has the potential to be involved in different intra-and extracellular signalling pathways in different chondrocytes cell types and may serve as a potential therapeutic target in degenerative and neoplastic diseases of cartilage. To further support the data presented in this thesis, which in turn, leads to further understanding of the role of NG2/CSPG4 in different diseases of the cartilage, the possible future directions would be important to consider.

6.2 Future directions

- The investigation of NG2/CSPG4 expression in a larger series of human chondrosarcoma biopsies, by immunohistochemistry and qPCR. This will provide an accurate quantification and comparison of NG2/CSPG4 expression in different grades of chondrosarcoma. In addition, a comparison of the expression level of NG2/CSPG4 in different types of chondrocytes can be done, both *in vivo* and *in vitro*.
- This study has shown that NG2/CSPG4 has altered patterns of expression and different distribution in different chondrocytic cells used. NG2/CSPG4 is expressed as a core protein only (270kD) in both JJ012 and C₂₀A₄ chondrocyte cell line, while multiple forms of NG2/CSPG4 including high molecular weight (GAG chain) forms up to 400kD and 270kD core protein were demonstrated in human OA chondrocytes.

It has been shown that the GAG chain of NG2/CSPG4 may/may not be required for the interaction of NG2/CSPG4 with different ECM molecules in different cell types. For

example, it has been shown that the presence of chondroitin sulphate chain is not necessary for NG2/CSPG4 binding to type VI collagen (Nishiyama et al 1996). However, this may have its functional implications on the interaction of NG2/CSPG4 with different cellular proteins, including ECM molecules. This, in turn, affects and involves in the mechanisms of different diseases. The roles of ECM molecules and its functional interactions with different membranous proteins have been thoroughly studied in different disease conditions, including cancers (Grossmann review 2002, Molecular mechanisms of “detachment-induced apoptosis—Anoikis).

It is possible that different forms and expression of NG2/CSPG4 in the different chondrocytic cells used may indicate different functional roles of this molecule in different diseases: OA and chondrosarcoma. It might be also useful to investigate the impact of the expression of multiple forms of NG2/CSPG4, the presence and absence of GAG chain of NG2/CSPG4 in OA chondrocytes (but not in chondrosarcoma) on the expression pattern and the distribution of different ECM molecules. This can be done by double immunofluorescence staining for both anti-NG2 antibodies and any ECM molecules: Collagen I, II, V, VI and FN. This should be followed by further analysis to assess the degree of correlation using Pearson coefficient or R total, the two threshold Manders percentages (tM1 and 2) together with the 2D histogram. The association of NG2/CSPG4 with collagen VI and thrombopoietin in OA chondrocytes but not in JJ012 grade II chondrosarcoma may support all points mentioned above and further emphasise the importance of further studies in this aspect.

A high proportion of NG2 in the adult rat spinal cord is saline-soluble and represents the shed ectodomain of NG2. It has been found that this shedding process is

metalloproteinase dependent and this has its axon growth-inhibitory activity in the brain (Asher et al 2005). It is possible that the low molecular weight form of NG2 in OA chondrocytes may represent a proteolytic fragment of this molecule. However, further studies are required in this aspect, first by sequencing this form and then to investigate the possible shedder behind this. ADMATS 4 and 5, together with MMP3 and 13 play an important role in OA pathogenesis. If it is proven that one of these are the possible shedder, this will allow us to further understand the role of NG2 in this disease process. In addition, further studies may be essential for the co-localisation of NG2/CSPG4 with both ER and Golgi complex in OA chondrocytes. This includes: 1) accurate quantification of both cytoplasmic and membranous expression of NG2/CSPG4 in OA chondrocytes. 2) compare that with the NG2/CSPG4 expression in other chondrocyte cell types and 3) understanding the signalling pathways behind this. All of these may provide us with a better understanding of the disease processes in OA and chondrosarcoma.

- In view of the effect of NG2/CSPG4 on different cellular processes that have influential roles in cancer pathogenesis, clarifying the potential signalling pathways behind these responses would provide a clear understanding of the role of this molecule in the pathogenesis of human chondrosarcoma. Also, performing these experiments in the presence or absence of ECM may help us to understand further the mechanisms of the cartilage diseases OA and chondrosarcoma. Observations that there may be down regulation of NG2/CSPG4 expression in OA (Ashleigh et al 2009) and its over expression in some chemo-resistant tumours, including glioblastomas, most melanomas and some leukaemias (Schrappe et al 1991, Behm et al 1996, Chekenya et al 2002 and

Li et al 2003) indicate the importance of clarifying the routes by which NG2/CSPG4 expression is regulated. Potential routes would be through integrins.

- The effects of NG2/CSPG4 loss on different cellular genes, especially the ones which are involved in OA pathogenesis were novel findings. Microarray studies would be important in this field to explore further the role of NG2/CSPG4, not only in OA, but in other diseases of the cartilage including chondrosarcoma.

List of Publications, Presentations, Poster Presentations and Awards to attend meetings

Published papers and abstracts

1. "Therapeutic Molecular Targets in Human Chondrosarcoma". NSM Jamil, SEM Howie and DM Salter. Review paper published in the International Journal of Experimental Pathology, 2010, Volume 91, 387-393.
2. "The expression and function of NG2/CSPG4 in human chondrocytes". NSM Jamil, SEM Howie and DM Salter. Abstract published in the International Journal Of Experimental Pathology, Volume 93, Issue 4, Pages 243–304, A1–A31
3. British Society for Matrix Biology- Autumn 2011 Meeting Report. Nuor Jamil, Sally Dreger and Christos Gavriilidis, recipients of BSMB Reporter Bursaries. The meeting report was published in the International Journal of Experimental Pathology, Volume 93, Issue 4, Pages 243–304, and A1–A31.
4. "The development of a model to study NG2/CSPG4 function in chondrocytes". NSM Jamil, SEM Howie and DM Salter. Abstract published in the International Journal of Experimental Pathology, 2011, Volume92, A1-A38
5. British Society for Matrix Biology- Autumn 2010 Meeting Report. Matthew Gardiner, Stephanie Dakin, Douglas Dyer, Maan Al-Abbasi and Nuor Jamil, recipients of BSMB Reporter Bursaries. The meeting report was published in the International Journal of Experimental Pathology, 2011, Volume92, A1-A38.
6. "Differential Expression of NG2/CSPG4 in articular Chondrocytes transformed and Chondrosarcoma cell lines". NSM Jamil, SEM Howie and DM Salter. Abstract published in the International Journal of Experimental Pathology, 2010, Volume 91, A1-A42.

Presentations and Posters

1. "NG2/CSPG4 regulates aggrecanase and MMP expression in human chondrocytes". NSM Jamil, SEM Howie and DM Salter. Abstract presented at the World Congress on Osteoarthritis, Barcelona's International Convention Centre (CCIB), 26-29th/ 04/ 2012, Barcelona, Spain. It is also accepted for poster presentation at the European Calcified Tissue Society meeting, 39th annual congress, 19-23th/05/2012, Stockholm, Sweden.
2. "The expression and function of NG2/CSPG4 in human chondrocytes". NSM Jamil, SEM Howie and DM Salter. Poster presentation at the BSMB meeting" Cell matrix interaction in health and diseases", 08-09/09/2011, Newcastle, UK.

3. "Development of a model to study NG2/CSPG4 roles in human chondrocytes". NSM Jamil, SEM Howie and DM Salter. Poster presentation at the Biennial Meeting for the American Society of Matrix Biology conference, 24-27/10/2010, Charleston, SC, USA.
4. "Differential Expression of NG2/CSPG4 in articular chondrocytes and chondrocyte cell lines". NSM Jamil, SEM Howie and DM Salter. Poster presentation in "Get Connected" conference, the Wellcome Trust Centre for Cell matrix Research Conference, 13th-15th /09/ 2010, University of Manchester, UK.
5. "The development of a model to study NG2/CSPG4 function in human chondrocytes". NSM Jamil, SEM Howie and DM Salter. Poster presentation in "Inflammation Meets Matrix Biology" BSMB Autumn Meeting, 6th-7th /09/2010, Norwich, UK.
6. "Differential Expression of NG2/CSPG4 in articular chondrocytes and transformed and Chondrosarcoma cell lines". NSM Jamil, SEM Howie and DM Salter. Poster presentation in "Vascular Matrix in Health and Disease "BSMB spring meeting and AGM, 29th-30th/09/2010, University of Manchester, UK.

Awards

1. British Society of Matrix Biology bursary award to attend the European Calcified Tissue Society Meeting, Stockholm, Sweden 2012 on 18/05/2012.
2. Pathology Society of Great Britain and Northern Ireland bursary award to attend the European Calcified Tissue Society Meeting, Stockholm, Sweden 2012 on 18/05/2012.
3. British Society of Matrix Biology bursary award as a reporter for the BSMB meeting, Newcastle 2011 on 08/09/2011.
4. British Society of Matrix Biology bursary award to attend Get Connected meeting, Manchester 2010 on 13/09/2010.
5. British Society of Matrix Biology bursary award as a reporter for BSMB meeting, Norwich 2010 on 06/09/2010.

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Appendix I

All reagents and tissue culture media were supplied by came from Sigma, UK unless otherwise stated.

Solutions and chemical reagents used in this study.

A. Solutions used in isolation and culture of chondrocytes

Anti-microbial solution

100ml of solution was prepared from 85ml sterile PBS, 5ml of Penicillin- Streptomycin (both 10000 IU/ml), 5ml L-Glutamine (200mM) and 5ml Fungizone (250µg/ml) (all from Invitrogen, UK).

Collagenase

Type H Collagenase (Sigma) was prepared from 1g Collagenase in 330ml Serum free Iscoves media (Sigma, UK). 20 ml aliquots were sterile, filtered and stored at -20oC.

Seeding media

Iscove's media (Sigma, UK) supplemented with 10% fetal calf serum (Firstlink), 10000 IU/ml Penicillin/Streptomycin, and 200mM L- Glutamine (Invitrogen, UK) and 2.5µg /ml Fungizone (Invitrogen, UK)

Feeding media

Iscove's media (Sigma, UK) supplemented 10% fetal calf serum (Firstlink), 10000 IU/ml Penicillin/Streptomycin, 200mM L- Glutamine (Invitrogen, UK), and 2.5µg /ml Fungizone (Invitrogen, UK)

Dulbecco's Modified Eagle's Media (DMEM)

DMEM media (Sigma, UK) supplemented with 10% fetal calf serum (Firstlink), 10000 IU/ml Penicillin/Streptomycin, and 200mM L- Glutamine (Invitrogen, UK) and 2.5µg /ml Fungizone (Invitrogen, UK).

B. Solutions used in whole cell lysis

Activated Na₃VO₄

A 200mM solution of Na₃VO₄ was prepared by dissolving 367.8 mg in 10ml d H₂O. The pH was adjusted to pH10 and the solution turned yellow. The solution was boiled until it was colourless, and then cooled. The solution was re-adjusted to pH10, boiled again and this process was repeated until the solution remains colourless at pH 10. The solution was liquored and stored at -20C°.

Wash buffer

1mM of activated Na₃VO₄ was prepared by diluting 1/200 in PBS from 200mM Na₃VO₄ stock solution. This solution was kept on ice.

Lysis buffer

A 10ml solution was prepared from 1ml of 10% Igepal (Sigma, UK), 50µl of 200mM Na₃VO₄, one complete protease inhibitor cocktail tablet (Roche) in PBS. This solution was kept on ice.

SDS lysis buffer

Was prepared from the following:

Lysis Buffer: - Mix one part Lysis Buffer I with two parts of Lysis Buffer II (ex:

1ml lysis buffer I + 2ml lysis buffer II)

Lysis Buffer I: 5% SDS; 150mM TRIS/Cl pH 6, 7; 30% Glycerol.

Lysis Buffer II: 25mM TRIS/Cl pH 8.2; 50mM NaCl; 0.5% NP – 40; 0.1% SDS;
0.1% Na Azide.

C. Solutions used for Lowry determination of the protein concentration

0.1N NaOH

Prepared from 4g NaOH in one litre of dH₂O

BSA (bovine serum albumin) Standards

A stock solution prepared from 1mg of BSA in 1ml of dH₂O. This solution was diluted with 0.1N NaOH to make the standards using the volumes in the table below.

:

Final BSA concentration μg/ml	Volume 1mg/ml BSA (μL)	Volume 0.1N NaOH (μL)
0	0	200
10	2	198
25	5	195
50	10	190
75	15	185
100	20	180
150	30	170
200	40	160

D. Protein standards for BCA assay

BSA standards prepared from a 2mg/ml stock solution provided in the kit diluted in PBS.

Protein standards used were prepared using the following volumes:

Vial	Concentration μg/ml	Volume of diluent (PBS) (μl)	Volume and source of BSA (μl)
A	2000	0	100 of stock
B	1500	41.5	125 of stock
C	1000	108.5	108.5 of stock
D	750	58.5	58.5 of vial B dilution
E	500	108.5	108.5 of vial C dilution
F	250	108.5	108.5 of vial E dilution
G	125	108.5	108.5 of vial F dilution
H	25	133.5	33.5 of vial G dilution
I	0 BLANK	133.5	0

F. Master mix for Reverse Transcription

REAGENT	VOLUME (n)	CONCENTRATION
5X RT buffer	4 μl	1X
dNTP mix: 10mM each of: dATP, dCTP, dGTP, dTTP	2 μl	1mM each: dATP, dCTP, dGTP, dTTP
20μM Oligo Dt	1μl	1μM
RNAse inhibitor	0.2μl	0.04U / μl
RT (enzyme)	1μl	1X

G. Master mix for PCR

Composition and concentration of reagents in the master mix.

Reagent	Volume (n)	Final concentration
10 x RT buffer	2µl	1X, 2.5 mM Mg ²⁺
D NTP	1µl	500µ M each dNTP
Primer mix *	0.4µl for GAPDH and 0.5 µl for NG2	
RT (enzyme)	0.1µl	0.8 U/ 20µl reaction
RNAse free water	14.5µl	

*Made up from 40µl each primer stock solution (50nmol in TE buffer; F, R or 1, 2) +20µl of RNAse free water.

H. Solutions used in Agarose gel electrophoresis

Tris Borate EDTA Buffer (TBE)

A 4L of 2X TBE was prepared by mixing one sachet of TBE buffer (MP bio chemicals, UK) in 4 litres of Milli Q water.

1X TBE was prepared by mixing equal amounts of 2X TBE and Milli Q water

1% Agarose gel

The gel was prepared by mixing 1.5g agarose (Sigma, UK) and 150ml 1X TBE buffer.

I. Solutions used in indirect immunofluoresence

1:1 Acetone / methanol solution

A 50ml solution of was prepared by mixing equal volumes (25ml) of acetone (Fisher scientific, UK) and Methanol (Fisher Scientific). This solution was prepared in advance and kept in the freezer to allow cooling down.

10% Goat serum

Prepared by mixing 1ml Goat serum and 9ml PBS.

The solution should be prepared fresh each time and sieved to remove any debris.

Propidium Iodide

Prepared from 1µl of the stock solution (1mg/ ml) and 10ml PBS.

J: Solutions used in western blotting

Materials	Amount for one gel	Amount for 2 gels
Transfer buffer	15mls	15mls
Anti oxidant	300µl	300µl
Methanol	30ml	60mls
Milli Q water	254.7mls	224.7mls

K: solutions used in flow cytometry

FACS wash buffer

Was prepared from BPS, 1%BSA and 0.05% sodium aziade.

L: solutions used in immunoprecipitation, in-gel digestion and mass spectrometry

0.25%coomassie blue stain solution

Was prepared from: 0.1g coomassie blue (Sigma, UK), 45mls methanol, 45mls distilled water and 10ml acetic acid).

Destaining solution

Was prepared from: 30ml methanol, 30ml acetic acid and 240ml distilled water.

Washing solution

Was prepared from: 200mM of NH_4HCO_3 (ABC) in 50% acetonitrile (ACN).

Reducing solution

Was prepared from: 20mM DTT, 200mM ABC and 50% ACN.

Alkylating solution

Was prepared from: 200mM ABC, 50mM Iodoacetamide (IAA) and 50% ACN.

Matrix used in MALDI protein spotting

Sinapinic acid (3, 5-dimethoxy-4-hydroxycinnamic acid) solution (10mg/ml) for proteins

Was prepared as follows:

1. Weigh 10mg sinapinic acid, which is stored at RT.
2. Add 400ul water and mix.
3. Add 100ul 3% TFA and mix.
4. Add 500ul ACN and mix.

5. Spin 5,000rpm 1min.
6. Use the supernatant for spotting with sample on the MALDI plate.

M: solutions used in cell adhesion

1% methylene –blue (20ml)

Was prepared from: 20g methylene blue in 20ml distilled water. Filtered sterilised.

Blocking solution (heat denatured BSA, 10mg/ml)

Was prepared as follows:

1. Weigh 0.5g BSA.
2. Add 25ml distilled water.
3. Shake and dissolve.
4. Put in pre-warmed water bath at 85°C for 10 minutes.
5. Cool down for few minutes. Dilute and use as required.

N: solutions used in inverse invasion assay

4µM Calcein (Invitrogen, UK)

Was prepared from: 350µl DMSO used to re-suspend the contents of 1tube. Used at 1µl/n of sample.